The Induction of Subsets of MUP Genes in the Liver by Different Patterns of GH Administration

by

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DECLARATION

This thesis was composed by the author and the work described is original work and was carried out by the author.

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ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
BISACRYLAMIDE	N,N,-methylene bis acrylamide
cDNA	complementary deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
dH_2O	distilled water
DTT	D,L-dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
GFC	Glass microfibre filters
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
IEF	Isoelectric focusing
IPTG	Isopropyl-β-D-thiogalactopyranoside
KLENOW	Large fragment Mr 68,000 of *E.Coli* DNA polymerase I
MB3	Mixed bed ion exchange resin
NP40	Nonidet-P40
OD	Optical Density

OLB	Oligo labelling buffer
OLIGO (dT)	Oligo (deoxythymidylic acid)$_{10-12}$
PAGE	Polyacrylamide gel electrophoresis
PEG	Poly ethylene glycol
POLY (A)	Poly (adenodylic acid)
POPOP	1,4-Di-[2-(5-phenyloxazoyl)]-benzene
PPO	2,5-Diphenyloxazole
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
tRNA	transfer ribonucleic acid
RNASE	ribonuclease
RNASIN	ribonuclease inhibitor
rNTP	ribonucleotide triphosphate
SDS	Dodecyl sodium sulphate
TCA	Trichloroacetic acid
TBE	Tris-Borate, EDTA buffer pH 8.3 (10x 108g Tris, 55g Boric acid, 9.3g EDTA per litre)
TE	10mM Tris, 1mM EDTA pH 7.5 - 8.0
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane
X-GAL	5-Bromo-4-Chloro-3-indolyl-β-D-galactoside

alf	mouse locus on chromosome 7; factor indicated by the albino lethal mutation
APF	transcription factor; albumin promoter factor
APO-AI	apolipoprotein AI
ARP-1	transcription factor; apolipoprotein AI regulatory protein
Balb/c	inbred strain of mice
BAP	transcription factor; B Activator Protein
bp	base pairs
C-P450$_{16\alpha}$	cytochrome P450
C57BL/6	inbred strain of mice
cAMP	cyclic adenosine monophosphate
C/EBP	transcription factor; CAAT / enhancer binding protein
DBP	transcription factor; D binding protein
EGF-R	epidermal growth factor receptor
ERE	DNA sequence; estrogen response element
fos	nuclear protooncogene
GH	growth hormone
GH-BP	GH binding protein
GH-R	GH receptor
bGH	bovine GH
hGH	human GH
oGH	ovine GH
rGH	rat GH
GP130	glycoprotein 130

GRE	DNA sequence; glucocorticoid response element
GRF	growth hormone releasing factor
HNF-1	transcription factor; hepatocyte nuclear factor - 1. Similarly HNF-3, HNF-4 and HNF-5.
vHNF-1	transcription factor; variant HNF-1
HP-1	transcription factor; hepatocyte specific promoter element.
HPT	hypothyroid
HPX	hypophysectomized
3ß-HSD	3ß-hydroxysteroid dehydrogenase
IIC11	cytochrome P450 IIC11. Similarly IIC12, IIIA2, IIC13
IGFI-R	insulin-like growth factor-I receptor
IL6-R	interleukin-6 receptor
IL1-R	interleukin-1 receptor
jun	nuclear protooncogene
LAP	transcription factor; liver-enriched transcriptional activator protein
LF-B1	transcription factor; liver factor B1
MUP	major urinary protein
mup-1	MUP locus on mouse chromosome 4
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PDGF-R	platelet-derived growth factor receptor
POU	Region of sequence similarity found in a

	group of transcription factors which includes Pit-1, Oct-1, Oct-2 and the product of the *unc-86* gene of *Caenorhabditis elegans*.
PRL	prolactin
PRL-R	PRL receptor
rPRL	rat PRL
raf	mouse locus;(*Afr-1*) regulation of α-fetoprotein
RI strains	recombinant inbred strains
Rif	mouse locus; (*Afr-2*) regulation of induction of α-fetoprotein
SS	somatostatin
TA	thyroid ablated
THX	thyroidectomized
Tse-1	mouse locus on chromosome 11; tissue-specific extinguisher-1

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Abstract

Group 1 major urinary protein (MUP) genes can be divided into subgroups named *Mup-1.1A*, *Mup-1.1B*, *Mup-1.1C* and *Mup-1.1D*. Allelic genes in Balb/c and C57BL/6 inbred strains of mice are referred to as *Mup-1.1A*^{a} and *Mup-1.1A*^{b} respectively. Expression of MUP genes in the liver is sexually dimorphic, however, the relative expression in males and females varies between subgroups and between allelic genes within a subgroup. Genes from two subgroups, *Mup-1.1A* and *Mup-1.1C* code for proteins which are synthesized at higher levels in male than female liver. In contrast, genes from two other subgroups, *Mup-1.1B* and *Mup-1.1D* code for proteins synthesized in male liver but undetectable in female liver. The *Mup-1.1C* subgroup contains two single copy allelic genes, *Mup-1.1C*^{a} and *Mup-1.1C*^{b}. In C57BL/6 expression of *Mup-1.1C*^{b} in the liver of males is twice as high as females, whereas in Balb/c, expression of *Mup-1.1C*^{a} in males is seven to twenty times as high as females. Several genes in the *Mup-1.1B* subgroup differ between Balb/c and C57BL/6 mice, however, expression in the liver of some genes in this subgroup is the same in both strains and is approximately one hundred times higher in males than females.

In the livers of growth hormone (GH) deficient *lit/lit* C57BL/6 mice MUP mRNA levels are approximately one hundred fold lower than in phenotypically normal *lit/+* C57BL/6 males. The sexually dimorphic pattern of MUP expression is also absent. Administration of GH to male *lit/lit* mice by one regime caused a large increase in the levels of particular MUP mRNAs in the liver, whereas administration by another regime did not. Altering the GH regime affected some MUP mRNAs more than others.

INTRODUCTION

LIVER SPECIFIC GENE EXPRESSION

The liver is a widely used organ in the study of cell specific gene expression. This is largely due to its technical ease of isolation and its near homogeneity of cell type. As with any organ the liver's structure is an integral part of its function and this must be considered when model systems are designed for the study of liver specific gene expression.

The liver is made up of many lobules which surround central venules. Capillary branches of the hepatic artery and hepatic portal vein lie on the outer edge of each lobule. Blood from the hepatic portal vein and hepatic artery enters the hepatic sinusoids. There is only one layer of hepatocytes between sinusoids and the plasma is in close contact with these cells. The sinusoids feed the central veins which drain into the inferior vena cava. There are two main cell types in the liver, Kupffer cells and hepatocytes. Kupffer cells are derived from monocytes which enter tissues to become tissue macrophages. They form a secondary defence against bacteria. Hepatocytes are specialized liver cells which are derived from the endoderm and they are responsible for specialized functions of the liver which are listed in table I.1. The sides of hepatocytes *in vivo* are adjacent to either the sinusoids, bile duct or other hepatocytes.

During development immature hepatocytes become quiescent cells, however liver damage results in cell division (Shafritz *et al.*, 1990; Sobczak *et al.*, 1989). Following regeneration the hepatocytes again become quiescent cells. The pattern of hepatocyte gene expression changes during development. Some genes are expressed in the foetus but not in the adult, while others are switched on at birth or during postnatal

Table I.1 Functions of the liver

formation of bile
carbohydrate storage and release
formation of urea
synthesis of plasma proteins
many functions related to the transport and metabolism of fat
reduction and conjugation of adrenocorticoid and gonadal steroid hormones
detoxification of many drugs and toxins

development (Gluecksohn-Waelsch, 1987; Powell *et al.*, 1984; Derman, 1981; Camper *et al.*, 1989). Gene expression in hepatocytes is also altered during an inflammatory response called the acute phase (reviewed by Baumann *et al.*, 1989; Schreiber *et al.*, 1989). This is an acute change in gene expression which results in the synthesis of plasma proteins involved in minimizing tissue damage during inflammation and cell death.

The different patterns of liver specific expression often result from differential gene transcription and subsequentially changes in transcription factors. There are a number of liver specific transcription factors, the activity of which can be modulated by interaction with other transcription factors. Some of these are activated in response to hormonal stimulation.

Model Systems For Studying Liver Specific Gene Expression

Model systems used to characterize the molecular basis of liver specific gene expression are often limited in their ability to imitate the *in vivo* hepatocyte. The organization of the cells in the *in vivo* liver is in some way necessary for full expression of liver specific genes. When this supracellular organization is disrupted, the capacity of the cells to express these genes is reduced or even abolished. For example only limited liver specific gene expression occurs in hepatoma cells (Knowles *et al.*, 1980; Deschatrette and Weiss, 1974; Szpirer *et al.*, 1979) or primary hepatocyte cultures (Grieninger, 1983; Clayton and Darnell, 1983). Although more liver specific gene expression occurs when primary hepatocytes are grown on matrigel (Friedman *et al.*, 1989b; Schuetz *et al.*, 1988) or feeder cells (Fraslin *et al.*, 1985), a direct effect of the cell matrix on transcription has not yet been proved (Bissel *et al.*, 1990). Some liver specific genes have been studied in transgenic animals where the normal liver environment is maintained. A further advantage of transgenic animals compared with isolated cells in culture is that one can study the expression of the transgene in several tissues simultaneously (Idzerda *et al.*, 1989; Krumlauf *et al.*, 1985; Hammer *et al.*, 1987; Yan *et al.*, 1990; Al-Shawi *et al.*, 1988). Transgenic animals suffer from several disadvantages compared with cells in culture, the largest of which is the amount of time required to analyse a large number of gene constructs.

Transcriptionally competent liver nuclear extracts have been used to identify sequences which are important for the transcription of a number of liver specific genes (Gorski *et al.*, 1986). The information from functional studies in these extracts can be combined with gel-shift assays and *in vitro* and *in vivo* footprinting assays to derive a picture of the functionally active factors which can bind to the gene

of interest (Grayson *et al.*, 1989; Mirkovitch and Darnell, 1991). Nuclear extracts have a large advantage over transgenic animals because a number of deleted or mutated constructs of the gene of interest can be easily analysed.

Ultimately a combination of many systems may be needed to identify all of the elements involved in liver specific gene expression. For example 147bp of the albumin proximal promoter is sufficient to allow liver specific expression in nuclear extracts (Gorski *et al.*, 1986; Lichtsteiner *et al.*, 1987), whereas 12kb of the proximal promoter are required to allow expression of an albumin transgene in mice (Pinkert *et al.*, 1987). Expression was achieved in transgenic mice from a construct containing the -10kb far upstream element fused to the proximal element. This indicates that the sequence of the far upstream element was important and not its position relative to the transcription start site (Pinkert *et al.*, 1987). Using nuclear extracts, Vaulont *et al.*, (1989) were unable to achieve a hormonal response seen *in vivo*. The hormonal response element was located more than 300bp upstream of the TATA box and the inability of this element to work *in vitro* may be due to differences in the folding of DNA in nuclear extracts compared with *in vivo*.

Liver specific transcription factors can sometimes be identified by characterizing mice carrying mutations which affect some aspects of liver specific gene expression. The mutated genes are likely to have some role in liver specific gene expression. Mice carrying the mutant alleles *rif*, *raf* and *alf* have different timing of liver specific gene expression during development. Mice with the *rif* allele fail to express α-fetoprotein during liver regeneration (Abelev, 1971) whereas those with the *raf* allele continue to express the foetal genes α-foetoprotein, and H19 at birth (Olsson *et al.*, 1977; Pachnis *et al.*, 1984). Mice with the *alf*

allele fail to turn on a set of cAMP and glucocorticoid regulated enzymes at birth (Ruppert *et al.*, 1990). A particular gene product is likely to be absent or altered due to each of these mutations and this could be identified.

When hepatoma cells are fused to other cell types the expression of liver specific genes is switched off in a process called extinction. Extinction is controlled by genetic loci acting in *trans* and can be reversed if certain chromosomes are lost from the cell hybrids (Chin and Fournier, 1989; Petit *et al.*, 1986; Peterson *et al.*, 1985). A locus called *Tse 1* is responsible for extinction of some liver specific gene expression (Thayer and Fournier, 1989; Lem *et al.*, 1988). *Tse 1* is thought to interfere with the transcription of genes which respond to cAMP by interfering with the binding of transcription factors to the cAMP response element (Ruppert *et al.*, 1990; Boshart *et al.*, 1990).

Liver-Specific Transcription Factors

Using a combination of *in vitro* and *in vivo* analysis, a number of transcription factors involved in liver specific gene expression have now been characterized, isolated and in some cases cloned. The first of these to be cloned was C/EBP (Landschutz *et al.*, 1988). C/EBP is a liver factor which is also found in a number of other tissues. Its distribution in gut, lung, placenta, adrenals and liver led to the suggestion that it is involved in the regulation of lipid metabolism (McKnight *et al.*, 1989). The expression of C/EBP is developmentally controlled being maximum at birth and falling postnatally. Hence it could be involved in the developmental control of gene expression (Birkenmeier *et al.*, 1989). C/EBP is not found in dedifferentiated hepatocytes or preadipocytes, but is expressed only in differentiated

cells (Birkenmeier *et al.*, 1989; Christy *et al.*, 1989). In differentiating 3T3-F442A preadipocytes the expression of C/EBP occurs at the same time as genes specific to the differentiated phenotype (Christy *et al.*, 1989). This is consistent with the involvement of C/EBP in the control of transcription of genes expressed in differentiated cells.

In nuclear extracts, C/EBP acts as a positive transcription factor which stimulates transcription from promoters linked to an upstream C/EBP response element (Friedman *et al.*, 1989). When it is expressed in hepatoma cells it can activate specific liver genes (Friedman *et al.*, 1989). C/EBP binds to a wide range of different DNA sequences, through which it can activate transcription. Consensus sequences include CCAAT boxes and enhancer core sequences (Johnson *et al.*, 1987; Graves *et al.*, 1986). CCAAT boxes are found in the 5' regions of almost all genes and are bound by a family of CCAAT box binding proteins of which C/EBP is a member (reviewed by Johnson and McKnight 1989; Raymondjean *et al.*, 1988; Chodosh *et al.*, 1988). In order to activate transcription *in vivo* C/EBP may have to displace another CCAAT box binding protein.

The structure of C/EBP provides some clues to how it functions. It contains a leucine zipper structure which is essential for its activity (Christy *et al.*, 1989). Leucine zippers are involved in dimerization and C/EBP can form homodimers and also heterodimers with other leucine zipper proteins (Roman *et al.*, 1990). Binding sites for C/EBP are found adjacent to AP1 sites which are binding sites for *fos- jun* heterodimers (reviewed by Johnson and McKnight, 1989). *Fos* and *jun* are also leucine zipper transcription factors which may interact with C/EBP and modulate its activity. A leucine zipper protein related to C/EBP was activated during the acute phase response (Akira *et al.*, 1990).

The second liver specific transcription factor to be cloned was HNF-1 also called LF-B1, APF and HP-1 (Frain *et al.*, 1989; Courtois *et al.*, 1987; Hardon *et al.*, 1988; Monaci *et al.*, 1988; Cereghini *et al.*, 1988; Schorpp *et al.*, 1988). HNF-1 mRNA was detected in kidney, intestine, spleen and thymus and functionally active protein was detected in nuclear extracts from liver and kidney (Frain *et al.*, 1989; Baumheuter *et al.*, 1990). HNF-1 activates a large number of liver genes and is expressed in differentiated but not dedifferentiated hepatocytes (Cereghini *et al.*, 1988; Baumheuter *et al.*, 1988). HNF-1 acts as a positive transcription factor by binding to a highly conserved inverted repeat sequence GTTAATNATTAAC (Courtois *et al.*, 1988). HNF-1 binding sites are invariably found within 150 base pairs 5' of the transcriptional start site (Courtois *et al.*, 1987; Cereghini *et al.*, 1988; Hardon *et al.*, 1988; Lichtsteiner *et al.*, 1987; Grayson *et al.*, 1988; Costa *et al.*, 1988; Tsutsumi *et al.*, 1989; Vaulont *et al.*, 1989; Frain *et al.*, 1990). The single known exception is a second site in the human albumin promoter between -360 and -344 (Courtois *et al.*, 1988). A single HNF-1 binding site within 150 base pairs of the transcriptional start site and a TATA box can direct liver specific expression in nuclear extracts (Ryffel *et al.*, 1989). The position seems to be important for the function of HNF-1. If the HNF-1 site is moved more than 400bp from the TATA box, no transcription occurs in nuclear extracts (Ryffel *et al.*, 1989). If the HNF-1 binding site in the albumin promoter was methylated by bacterial Dam-methylase, its affinity for HNF-1 was lowered. Binding of HNF-1 to methylated HNF-1 sites required cooperation of other *trans*-acting factors bound to adjacent upstream regions of the promoter (Tronche *et al.*, 1989).

HNF-1 is a member of a family of proteins which bind to the same consensus sequence (Rey-Campos *et al.*, 1991). A factor with the same binding specificity as HNF-1 was identified in dedifferentiated

hepatoma cells. This factor is called vHNF-1 (Cereghini *et al.*, 1988; Baumheuter *et al.*, 1988; Rey-Campos *et al.*, 1991) or LF-B3 (De-Simone *et al.*, 1991) and can form heterodimers with HNF-1. Both HNF-1 and vHNF1 are distant relatives of the homeodomain proteins which regulate changes in phenotype during development.

To date, eight different cDNAs or genes corresponding to liver specific transcription factors have been cloned (Table I.2). A number of other factors which are involved in liver specific regulation have been identified. These include BAP (Kugler *et al.*, 1990) and HNF-5 (Grange *et al.*, 1990).

In general the liver specific transcription factors share homologies with previously recognised structural components of transcription factors. These include zinc fingers, homeodomains, leucine zippers, basic regions and the POU domain (reviewed by Struhl, 1989). It is not known whether transcription factors with a particular structural domain have a particular role in gene regulation. For example factors with homeodomains may be important in development while factors with zinc fingers may be important in hormonal induction.

Zinc fingers are formed by the interaction of two pairs of cysteine residues or a pair of cysteine and a pair of histidine residues with a single zinc ion (reviewed by Evans and Hollenberg, 1988; Fairall *et al.*, 1986; Rhodes and Klug, 1986). These interactions generate a configuration called a zinc finger which binds to the DNA in a sequence dependent manner. The nuclear hormone receptors are zinc finger proteins, and there are also many "orphan" receptors with no known ligands amongst which are two liver transcription factors, Hepatocyte Nuclear Factor-4 (HNF-4) (Sladek *et al.*, 1990) and Apolipoprotein AI

Table I.2 Liver specific transcription factors.

Liver Factor	Structure	Reference
C/EBP	leucine zipper and basic	Landschulz *et al.*, 1988 Birkenmeier *et al.*, 1989
DBP	basic	Mueller *et al.*, 1990
HNF-1 (LF-B1, APF)	homeodomain, POU A domain	Frain *et al.*, 1989
HNF-3A	new type	Lai *et al.*, 1990
HNF-4	zinc-finger	Sladek *et al.*, 1990
LAP	leucine zipper and basic	Descombes *et al.*, 1990
ARP-1	zinc-finger	Ladias and Karanthanasis, 1991

Regulatory Protein 1 (ARP-1) (Ladias and Karathanasis, 1991). Zinc finger proteins are able to form homo- and hetero-dimers (reviewed by Forman and Samuels, 1990) and some can also interact with leucine zipper proteins (Schule *et al.*, 1990; Yang-Yen *et al.*, 1990; Jonat *et al.*, 1990). These interactions may be important for hormone responses.

The homeodomain consists of a helix-turn-helix motif, two α-helices separated by a β-turn (Laughon and Scott, 1984; Shepherd *et al.*, 1984). The α-helices interact with a specific DNA sequence which is usually an inverted repeat suggesting that these proteins bind to DNA as dimers. Both homodimers and heterodimers can form and bind to DNA (Rey-

Campos *et al.*, 1991). The homeodomain proteins are expressed in different regions of the embryo during development (Manley and Levine, 1985; Gehring, 1985). A distant relative (HNF-1) is found amongst the liver transcription factors (Frain *et al.*, 1989). HNF-1 also contains a POU-A domain (Baumheuter *et al.*, 1990), a conserved domain found in a number of transcription factors which turn on gene expression during development (He *et al.*, 1989; Herr and Sturm, 1988; Rosenfeld, 1991). The POU-domain itself is important for DNA binding but may also be involved in interaction with other transcription factors.

Leucine zippers are coiled coil protein structures through which two polypeptides bind to each other. The dimer is able to bind to a specific DNA sequence (Landshulz *et al.*, 1988). The region of the polypeptide which forms the coiled coil contains four or five leucine residues which are spaced at seven residue intervals. The DNA binding domain is in another part of the protein and is often basic but can be of other types (reviewed by Busch and Sassone-Corsi, 1990). Many leucine zipper proteins are activated in response to mitogens like polypeptide hormones (reviewed by Herschman, 1989). Others are inactivated or modified in a similar way in response to unknown stimuli. In general, these proteins may be involved in mediating hormone responses. In the liver, two leucine zipper transcription factors have been identified, C/EBP (Landshulz *et al.*, 1988) and Liver Activation Protein (LAP) (Descombes *et al.*, 1990). These proteins may be members of a family of leucine zipper proteins which are activated in the liver at different times (Akira *et al.*, 1990; Roman *et al.*, 1990).

Liver specific transcription factors are also synthesized in other tissues, mRNA for four out of six of the cloned factors where tissue distribution has been examined, are found in kidney and intestine. mRNA

for three out of six are found in lung, spleen and brain (references as table I.5). Some genes which are expressed in the liver are also expressed in these other tissues. Their expression is controlled by the level of both transcription factors and inhibitors. The presence of mRNA for transcription factors does not always correlate with the presence of the active factor. Some of the transcription factors are regulated at levels other than transcription.

HORMONAL REGULATION OF GENE EXPRESSION IN THE LIVER

Much is known about the regulation of gene expression in the liver by several different types of hormone. Here the hormones are divided into two groups. The first group consists of hormones which can pass through the cell membrane and bind to intracellular or nuclear receptors. These receptors can bind directly to DNA to cause transcriptional activation (reviewed by Beato, 1989; Auricchio [illegible], 1989; Beato *et al.*, 1989; Gehring [illegible], 1987; Evans, 1988; Forman and Samuels, 1990). The second group are polypeptide hormones which cannot enter the cell directly but which bind to membrane bound receptors. A number of intracellular pathways convey the signals from the receptor to intracellular and intranuclear factors (reviewed by Kahn, 1990; Ullrich and Schlessinger, 1990; Cosman *et al.*, 1990).

Both groups of hormones have "liver specific" effects on gene expression. In some cases liver specific transcription factors bind to regions of the DNA adjacent to the hormone response elements and interact with the hormone-activated transcription factors. Interactions of this sort appear to be an important component of hormone-regulated

gene expression.

In the liver many genes are subject to hormonal regulation. For example, many of the genes involved in carbohydrate metabolism are controlled by adrenal steroids and insulin (Jacobs and Cuatrecasas, 1981; Kahn *et al.* 1981). Fat metabolising enzymes are controlled by adrenal steroids, thyroxine and insulin (Elshourbagy *et al.*, 1985; Strobl *et al.*, 1990; Apostolopoulos *et al.*, 1988). Acute phase reactants are controlled by adrenal steroids, interleukins and interferons (Baumann *et al.*, 1989; Schreiber *et al.*, 1989). Proteins involved in sexually dimorphic steroidogenesis and drug metabolism are regulated by GH and thyroxine (Porsch-Hallstrom *et al.*, 1990; Zhao *et al.*, 1990; Jansson *et al.*, 1988; Johansson *et al.*, 1989; Oscarsson *et al.*, 1991; Shapiro *et al.*, 1989; Norstedt and Palmiter, 1984). None of these responses is regulated exclusively by a single hormone and interactions between hormone-activated transcription factors is anticipated. The molecular details of some hormone stimulated effects on liver gene expression are described in the following sections.

Nuclear Hormone Receptors

Receptors for all of the diffusible hormones have now been cloned and they are members of a large superfamily of molecules. The family includes "orphan" receptors for which there is no known ligand (Ladias and Karanthansis, 1991). The receptor is able to bind specifically to the ligand and to a region of the DNA (the Hormone Response Element HRE). This binding activates or inhibits transcription.

The sequences to which receptors bind have been "idealized" into two core motifs 6bp long either TGACCT (thyroxine, retinoic acid and

oestrogen) or TGTTCT (glucocorticoids). HREs normally contain a number of core motifs and the orientation and spacing of these motifs in the DNA determines the specificity of the HRE. Different combinations of core motifs can be assembled which mediate responses to different subsets of hormones (Naar *et al.*, 1991; Umesono *et al.*, 1991). The responses can be either positive or negative or a combination. For example two direct TCAGGTCA repeats separated by 3bp can confer a retinoic acid response whereas two inverted repeats of the same sequence separated by 3bp confer an oestrogen response. Two adjacent inverted repeats of the same sequence can confer a retinoic acid, thyroxine and vitamin A response (Naar *et al.*, 1991; Umesono *et al.*, 1991).

The DNA binding domain of the receptor polypeptides are cysteine rich and form two zinc fingers which recognize and bind to the HRE. Amino-acids at three positions in the first zinc finger are important for discrimination between the core motifs and are referred to as the discriminatory amino-acids (reviewed by Forman and Samuels, 1990). Some receptors are very stringent in their recognition of core motifs. These include the oestrogen and glucocorticoid receptors which only recognise inverted repeats of their respective core sequences. Substituting the three discriminatory amino-acids of the glucocorticoid receptor for the three discriminatory amino-acids from the oestrogen receptor generates a mutant receptor which recognises a GRE but not an ERE (Mader *et al.*, 1989).

Other receptors including the thyroxine and retinoic acid receptors can bind as dimers to both direct and inverted repeats of their preferred core sequence. The C-terminal region of these receptor proteins is important for dimerization and it may be a flexible region which can move around to allow dimerization irrespective of orientation, alterna-

tively, each receptor protein may have more than one dimerization face (Naar *et al.*, 1991; Umesono *et al.*, 1991).

Ligand binding causes an alteration in the conformation of the receptor. This is important for many activities of the receptor polypeptides for example DNA binding, transactivation, association with other proteins, dimerization and nuclear localization. The function of the receptor is affected not only by the concentrations of receptor and ligand but also by other proteins which may bind to the receptor, the HRE or the ligand. Other proteins can activate or inactivate the receptor and in this way modify the response in a cell-specific manner. In the liver the direct interaction of receptors with liver specific transcription factors have been demonstrated (Grange *et al.*, 1990). In other cases liver specific transcription factors are necessary for transcription but may not interact directly with the receptor.

Membrane Bound Receptors

Membrane bound receptors for many polypeptide hormones have been cloned. These are classified into several groups based on their structural homologies. The two major groups found in the liver are described here. The first group have an integral tyrosine kinase activity and include the EGF-R, Insulin-R, IGFI-R and PDGF-R (Ullrich and Schlessinger, 1990). The second group belong to the hematopoietin receptor superfamily and include IL6-R, IL1-R, GH-R and PRL-R (Cosman *et al.*, 1990). Both families of receptors have an extracellular glycosylated ligand binding domain, a single hydrophobic transmembrane domain and an intracellular domain. Amongst the receptors with tyrosine kinase activity there are several structurally distinct types of extracellular domain. The tyrosine kinase component of the intracellu-

lar domain, however, is very highly conserved. The members of the hematopoietin receptor superfamily have no single recognised homology in the intracellular domain. The characteristic features of this family are two conserved pairs of cysteines and a Trp-Ser-X-Trp-Ser motif in the extracellular domain (Goodwin *et al.*, 1990).

In the cell membrane there are frequently two forms of a particular receptor with different affinities for the ligand (Gearing *et al.*, 1989; D'Andrea *et al.*, 1989; Yamasaki *et al.*, 1988; Wang *et al.*, 1989). This difference in affinity is due to specific binding sites with two different binding affinities, as opposed to specific and non-specific sites. The number of high affinity sites is small compared with low affinity sites. The biological activity of the hormone is consistent with the high affinity sites being the active receptors (D'Andrea *et al.*, 1989) which suggests that they are modified in some way to increase their affinity for the ligand. The modification of receptors is cell specific. When a receptor cDNA clone is expressed in different cell types, some produce both high and low affinity sites while others produce only low affinity sites (Gearing *et al.*, 1989). Changes in the affinity of receptors can be brought about by post-translational modifications such as glycosylation (Szecowka *et al.*, 1990) or phosphorylation or in some cases by interaction with another protein (Hibi *et al.*, 1990).

Association of some receptors with proteins thought to be part of the secondary messenger pathway, such as GTPases (G proteins) and tyrosine kinases, increases their affinity for the ligand. The receptor domains responsible for this interaction have not been characterized. The G-proteins, which are intracellular, associate with the cytoplasmic or transmembrane domain of the receptor. Tyrosine kinases may be intracellular or membrane-bound proteins. Soluble versions of many membrane

receptors have been identified which can be formed from the membrane-bound receptor by peptide cleavage or coded for by a separate RNA (Petch *et al.*, 1990). A tyrosine kinase called GP130 can interact with both the membrane bound IL6-R and the soluble IL6-R. The soluble receptor lacks both the transmembrane and cytoplasmic domains which means that GP130 can interact with the extracellular domain (Hibi *et al.*, 1990). The soluble IL6-R can potentially interact with GP130 in a cell with no endogenous IL6-R.

Receptors act through secondary messenger pathways which have not been fully characterized for any receptor. There are several pathways which the same receptor may activate to differing extents (Kahn, 1989; Ullrich and Schlessinger, 1990). In all pathways phosphorylation of proteins on tyrosine, serine or threonine residues plays a central role. There are a large number of protein kinases and phosphatases which are activated or inactivated by different receptors amongst which are four classes; tyrosine kinase, phospholipase, protein kinase C and cAMP dependent protein kinase. Tyrosine kinase phosphorylates tyrosine residues of a number of intracellular substrates and many receptors themselves have tyrosine kinase activity. Phospholipase C-gamma is activated by a tyrosine kinase. It can then hydrolyse inositol phospholipids to generate inositol tri-phosphates and diacylglycerol. Protein kinase C is activated by diacylglycerol and phosphorylates serine and threonine residues. cAMP dependent protein kinase is activated by cAMP generated from activation of adenylate cyclase by G-proteins. cAMP dependent protein kinases phosphorylate proteins on serine residues.

Each receptor, with or without an integral tyrosine kinase, activates a different subset of protein kinases to generate many different secondary messenger pathways using different subsets of the same basic

components. This ultimately leads to changes in protein activity and gene expression. As one would expect, some transcription factors are activated by phosphorylation.

Transcription factors whose activity is altered by peptide hormones have been isolated from stimulated cells by their ability to bind regions of DNA known to mediate the hormonal response. The genes or cDNAs corresponding to some of these factors have been cloned. Some have been shown to interact with liver specific transcription factors (Sladek *et al.*, 1990; Akira *et al.*, 1990) and some are phosphorylated in response to hormonal stimulation (Kessler *et al.*, 1990; Imam *et al.*, 1990). To date, only a very small number of peptide hormone stimulated transcription factors have been identified. There is still a long way to go before the response to peptide hormones has been fully characterized.

THE PRODUCTION OF GROWTH HORMONE AND THE CONTROL OF SEXUAL DIMORPHISM IN THE LIVER

The following section contains a description of GH, its synthesis in somatotrophs and the timing and pattern of GH release. It also includes a discussion of the control of GH production and release by somatostatin (SS), growth hormone releasing factor (GRF), the sex steroids, adrenal and thyroid hormones. The final section relates to regulation of sexual dimorphism in the liver by GH.

GH Synthesis, The Timing and Pattern of GH Release

GH genes and cDNAs from a number of species including human, rat, cow,

pig, chicken and horse have been cloned (Goeddel *et al.*, 1979; Seeburg *et al.*, 1977; Seeburg *et al.*, 1983; Lamb *et al.*, 1988; Miller *et al.*, 1980). GH is closely related to prolactin (PRL). The genes for these two hormones probably arose by gene duplication and divergence. hGH can cross react with both the rGH and rPRL receptors (Herington *et al.*, 1983) and it is important to distinguish between the lactogenic and somatogenic properties of GH when it is administered across species. Both PRL and GH are made in the pituitary gland. During development stem-somatotrophs are the common precursor to both the GH-producing somatotroph and PRL-producing lactotroph populations (Behringer *et al.*, 1988; Borrelli *et al.*, 1989). Transcription of both PRL and GH is activated by a pituitary specific transcription factor *pit-1* also called GH1. Other factors confine transcription of each hormone to its respective cell type during development (Simmons *et al.*, 1990; Fox *et al.*, 1990; Crenshaw *et al.*, 1989).

Plasma GH levels begin to increase in rats during the late fetal period then decline after birth. GH levels increase again before puberty and decrease in old age (Eden [illegible], 1979; Sonntag *et al.*, 1980). The levels of GH in the plasma of pubertal and adult rats vary episodically in a sexually dimorphic manner. Male rats have a very low or undetectable basal level of GH in the plasma which rises during regular spikes of GH release every 3 to 3.5 hours. Female rats have a high basal level of GH with frequent irregular spikes of GH release which are lower than those found in males and are slightly elevated during the night (Millard *et al.*, 1986; Saunders *et al.*, 1976).

Plasma levels of GH also vary in humans. The variation during development is similar to that in rats, however, in adult humans GH secretion is oscillatory rather than episodic. In both sexes GH pulses occur with an average frequency of 13 per day. In contrast to rats,

both the pulse amplitude and the basal secretion is higher in females (Winer *et al.*, 1990).

Control of GH Synthesis and Release by Thyroxine, Adrenal and Sex Steroids, Somatostatin (SS) and Growth Hormone Releasing Factor (GRF).

In gonadectomized rats the sexually dimorphic pattern of GH release is lost. Administration of androgens to gonadectomized males or adult females causes a masculinization of GH release by lowering basal GH secretion and increasing spike height (Ja nsson and Frohman, 1987). Administration of estradiol to gonadectomized female mice or adult males caused a feminization of GH release by increasing basal GH levels and GH pulse frequency and decreasing pulse amplitudes. Partial masculinization of the GH pattern was retained if male rats were gonadectomized during puberty. This indicates that neonatal androgens could partially imprint a male pattern of GH secretion on adults (Jansson *et al.*, 1985; Ja nsson and Frohman, 1987). Both adrenalectomy and thyroidectomy of rats resulted in reduction in the level of GH release which was restored by administration of glucocorticoids or thyroxine respectively (Spindler *et al.*, 1982).

The sex steroids and glucocorticoids influence both the release and synthesis of GH (Frohman *et al.*, 1987; Eden *et al.*, 1987; Vale *et al.*, 1983; Hertz *et al.*, 1989). The effect of the sex steroids on GH release and synthesis is partially indirect and mediated by two hypothalamic peptides, GRF and SS, which act antagonistically to regulate GH production and release. These two peptides are discussed further below. The sex steroids also act directly on the pituitary. Androgens increase the response to GRF and SS while oestrogens decrease the response (Eden

et al., 1987; Webb *et al.*, 1983; Simard *et al.*, 1986; Wehrenberg *et al.*, 1985; Fukata and Martin, 1986; Evans *et al.*, 1985).

The transcription of both rGH and hGH are influenced by a number of hormones which interact at various levels. Glucocorticoids increase transcription of both rGH and hGH. Thyroxine increases the transcription of rGH and bGH but not hGH (Brent *et al.*,, 1988). GRF increases transcription of all GHs. The mechanism of hormone action upon GH transcription is complex. Both glucocorticoids and thyroxine along with retinoic acid have a direct receptor mediated effect on rGH transcription. The effect of GRF on transcription is mediated by cAMP which may act by altering the binding of pituitary specific transcription factors to the rGH promoter. The many hormones act synergistically on GH transcription (Bedo *et al.*, 1989; Copp and Samuels, 1989; Dana and Karin, 1989).

The sex steroids regulate the production of GRF and SS by an unknown mechanism. GRF is a 40 to 44 amino-acid peptide and SS is a 14 amino-acid peptide (Brazeau *et al.*, 1973; Rivier *et al.*, 1982). GRF promotes GH synthesis and release and is a mitogen for somatotrophs (Vale *et al.*, 1983; Billestrup *et al.*, 1986; Billestrup *et al.*, 1987). SS inhibits the action of GRF. In male rats SS tone is high during the low basal release of GH (Clark and Robinson, 1987). When SS tone is high GRF administration does not lead to GH release (Clark and Robinson, 1987). SS inhibits GH release but allows some GH synthesis to occur. GH accumulates and in male rats is released in spikes in response to SS withdrawal and GRF pulses. SS and GRF levels are lower and more constant in female rats. GRF administration leads to GH release at any time but the response is lower than male spikes of GH release (Clark and Robinson, 1985). The effect of sex steroids on GH release can be mimicked by administering SS and GRF to imitate that of either a male

or a female (Clark and Robinson, 1987).

The Regulation of Sexual Dimorphism In The Liver By GH

In the liver many proteins which are regulated by GH are also sexually dimorphic (Table I.3A and B) and like the pattern of GH in the plasma, the pattern of gene expression of these GH responsive proteins changes during puberty. This indicates that the pulsatile male or the more continuous female patterns of plasma GH alter gene expression in the liver. Both patterns of plasma GH have both inhibitory and inductive effects on different substrates. Particular examples of proteins regulated in different ways by GH can be found in the Cytochrome P450 gene family. IIC11 and IIC12 are not expressed in the immature rat and at puberty expression of IIC11 is induced by the male pattern of plasma GH while the female pattern has no effect (Morgan *et al.*, 1985). In direct contrast to this IIC12 is induced at puberty by the female pattern of plasma GH while the male pattern has no effect (Guzelian *et al.*, 1988). IIC13 and IIIA2 on the other hand are expressed in the immature rat. At puberty expression is suppressed by both patterns of plasma GH but more by the female pattern than the male pattern (McClellan-Green *et al.*, 1989; Waxman *et al.*, 1989).

Other hormones such as insulin, thyroxine and glucocorticoids can enhance or repress expression of GH responsive genes in the liver (Tollet *et al.*, 1990; Mode *et al.*, 1989; Waxman *et al.*, 1990; Shimada *et al.*, 1989; Keller and Taylor, 1979). Part of the action of these hormones may be mediated by their effect on GH expression as mentioned above but in addition, direct effects of these hormones have been observed on GH responsive genes in isolated hepatocytes (Tollet *et al.*, 1990; Guzelian *et al.*, 1988). At least part of this effect is due to the action of thyroxine, glucocorticoids and insulin on elements of the GH

signalling pathway, for example on the GH receptor (Tollet *et al.*, 1990; Nimi *et al.*, 1990).

Table I.3A Sexually dimorphic gene expression in the liver

Genes expressed at higher levels in male liver		
	mouse	**rat**
Sex limited protein	Hemmenway and Robins, 1987	NA
EGF-R	Jansson *et al.*, 1988	Johansson *et al.*, 1989
MUP, α_{2u}- globulin	Finlayson *et al.*, 1963	Roy and Neuhaus, 1966
P450 IIC11	NA	Guzelian *et al.*, 1988
P450 IIC13	NA	McClellan-Green *et al.*, 1989
P450 IIA2	NA	Waxman *et al.*, 1989b
P450 IIIA2	NA	Shimada *et al.*, 1989
3β-HSD	NA	Zhao *et al.*, 1990
C-P450$_{16\alpha}$	Noshiro and Negishi, 1986	
c-myc	NA	Porsch-Hallstrom *et al.*, 1990

Table I.3B Sexually dimorphic gene expression in the liver

Genes expressed at higher levels in female liver		
	mouse	**rat**
P450 IIC12	NA	Mode *et al.*, 1989
I-P450$_{15\alpha}$	Noshiro and Negishi, 1986	NA
PRL-R	Norstedt and Palmiter, 1984	Robertson *et al.*, 1990
Apolipoprotein E	NA	Oscarsson *et al.*, 1991

GH receptor cDNAs have been cloned from rabbit, rat and mouse (Leung *et al.*, 1987; Mathews *et al.*, 1989; Smith *et al.*, 1989). The nucleic acid sequences of the receptors from different species share approximately 70% homology. The GH-Rs are homologous to other growth factor receptors and are members of the hematopoietic receptor gene superfamily (reviewed by Cosman *et al.*, 1990). The same or closely related GH-Rs are expressed in many cell types (Tiong and Herington, 1991) and expression is not sexually dimorphic in the liver (Herington *et al.*, 1983).

Several different sizes of receptor RNA and protein are found (Smith *et al.*, 1987; Spencer *et al.*, 1988). In rats and mice two of these are due to alternative patterns of splicing of the GH-R pre-mRNA at the exon 7 to 8 junction (Smith *et al.*, 1989; Baumbach *et al.*, 1989). The two sizes of mRNA produced in this way code for two proteins with the

same N-terminus. The longer protein (GH-R) has three domains, an extracellular ligand binding domain, a hydrophobic trans-membrane domain and a cytoplasmic domain. The shorter protein (GH-BP) has the same extracellular domain but has a substituted transmembrane domain and lacks the cytoplasmic domain (Mathews *et al.*, 1989; Baumbach *et al.*, 1989; Smith *et al.*, 1989). The role of the GH-BP is not known.

The extracellular domain is highly glycosylated which increases its affinity for GH (Szecowa *et al.*, 1990; Leung *et al.*, 1987). The intracellular domain has no homology to tyrosine kinase or any other signalling molecules (Mathews *et al.*, 1989; Leung *et al.*, 1987) however highly purified GH-Rs exhibit tyrosine kinase activity (Carter-Su *et al.*, 1989). Tyrosine-kinase associated GH-R is closely related to the cloned GH-R and may either be identical or a receptor isoform which has not been cloned (Stubbart *et al.*, 1991).

Following GH binding the GH-Rs are rapidly internalized. In male rats this results in a depletion of GH-R from the membrane shortly after GH pulses (Bick *et al.*, 1989). Both GH and the GH-R are rapidly processed and destroyed and replacement of GH-R on the membrane probably requires *de novo* synthesis (Baxter [illegible], 1985). It is not clear whether the GH receptor plays an active part in the reaction cascade or serves only to internalize the ligand.

Further studies are required in order to understand the response to the male and female patterns of plasma GH at the membrane. At present it is known that an interpulse interval of at least 2.5 hours is an essential component if the plasma profile is to be recognised as the male pattern. Neither the peak height nor width are important. After a pulse of GH the hepatocytes become refractory to further pulses for 2 to 2.5 hours and this can occur even with low pulses probably because

of the high affinity of the GH-R for GH (Leung *et al.*, 1987). This would be consistent with a model in which the interpulse interval is the time required for *de novo* synthesis of GH-Rs and their insertion into the membrane.

Further studies are also required to characterize the intracellular signals and molecular changes that lead to the two different patterns of gene expression in males and females. At present it is known that the stimulation of IGF-I transcription by GH occurs to the same extent in males and females (Maiter *et al.*, 1988) and that IGF-I mRNA increases within hours of GH stimulation (Tollet *et al.*, 1990; Johnson *et al.*, 1989), in the absence of protein synthesis (Tollet *et al.*, 1990). In contrast transcription of P450 and MUP are sexually dimorphic (Mode *et al.*, 1989; Derman, 1981), it can take several days for GH to induce P450 mRNA (Noshiro and Negishi, 1986) and the induction requires protein synthesis (Tollet *et al.*, 1990).

THE MOUSE MAJOR URINARY PROTEINS (MUPS)

Although a function has not been ascribed to MUPs and the closely related rat α_{2u}-globulins, they are probably involved in the transport of a small molecule (Held *et al.*, 1987). The expression of MUPs and α_{2u}-globulins are limited to tissues involved in secretion. Both are expressed in liver and the lachrymal, salivary, sebaceous glands, α_{2u}-globulin is also expressed in the preputial glands (Kuhn *et al.*,1984, Held *et al.*, 1989). High levels of MUP and α_{2u}-globulin are excreted in the urine and α_{2u}-globulin is found in saliva (Laperche *et al.*, 1983). MUPs and α_{2u}-globulin belong to a superfamily of proteins whose function is to transport small hydrophobic molecules like retinol (vitamin A), odorants and branch chain fatty acids. Members of the protein superfamily are characteristically small (160 to 180 amino-acids long), acidic and acid stable proteins. The ligand binding site is at the centre of a β-barrel and contains the hydrophobic residues tryptophan and phenylalanine. Members of the protein superfamily are commonly filtered out in the glomeruli and found in urine, particularly after tubule damage. Non-physiological binding of α_{2u}-globulins to two small synthetic hydrophobic molecules (d-limonene and 2,2,4-trimethyl pentane) found in petrol prevents degradation of α_{2u}-globulin in the nephrons and leads to kidney necrosis (Lehman-McKeeman *et al.*, 1989; Swenberg *et al.*, 1989).

The MUP proteins are 162 amino-acids long with an 18 to 22 amino-acid signal peptide. α_{2u}-globulins are 162 amino acids long with a 19 amino acid signal peptide. Laboratory mouse or rat strains secrete eight to twelve different MUPs or α_{2u}-globulins respectively in their urine which are heterogeneous in charge. The proteins are coded by different genes and single amino-acid differences lead to products distinguishable by narrow range isoelectric focusing (IEF). In addition to the

different charged MUP proteins there are at least three different sizes of MUPs distinguished by SDS PAGE. The higher molecular weight MUPs are variants which have an n-linked glycosylation site which is also present in α_{2u}-globulins at amino acid 35 (Uterman *et al.*, 1981). The structure of the lowest molecular weight MUPs is not known. It is also not known why there are so many different MUP and α_{2u}-globulin proteins. One could hypothesize that they are necessary to bind different ligands.

MUPs and α_{2u}-globulins form very large, closely related gene families. There are 30 to 35 MUP genes all located on chromosome 4 at a genetic locus called *Mup-1* (Hastie *et al.*,1979, Bishop *et al.*,1982, Krauter *et al.*,1982, Bennett*et al.*,1982, Shi *et al.*,1989). Similarly, there are 20 - 25 rat α_{2u}-globulin genes at the *Mup-1* locus on rat chromosome 5 (Kurtz, 1981). The MUP genes are arranged in head to head pairs, forming large imperfect palindromes (Bishop *et al.*, 1985, Ghazal *et al.*,1985). These are tandemly arranged but their relative orientations are not known (Bishop *et al.*,1985). The arrangement of the MUP genes may explain their number. Large inverted repeats in DNA are often associated with the tandem amplification of genes under selective pressure and similar mechanisms may underlie amplification in normal cells (Ford and Fried, 1986).

The total size of the MUP palindrome is 45kb, with a central unique region of 6kb and 5kb of 5' sequence, 3.5kb of coding region and 11kb of 3' sequence duplicated in divergent orientations on either side (Bishop *et al.*, 1985, Ghazal *et al.*,1985). Two groups of MUP genes, group 1 and group 2 were defined by sequence homology and each palindromes contains a group 1 and a group 2 gene. All of the group 1 genes which have been isolated appear to be functional whereas the group 2 genes are pseudogenes with a conserved stop codon at amino-acid 7 in

the reading frame used by group 1 genes. A number of pseudogenes were found amongst α_{2u}-globulin genomic clones suggesting that α_{2u}-globulin genes may have the same organisation as MUPs (Winderickx *et al.*, 1987). Genes within group 1 or group 2 are very similar to each other, based on the coding region sequences (1 to 2% difference between group 1 genes) (Ghazal *et al.*, 1985). Between the groups there is a difference of 11 to 15% spread equally over the introns and exons. This suggests that the group 2 genes arose as a duplication of a pseudogene allowing equal divergence of the coding and non-coding region (Clark *et al.*, 1985). Both groups have diverged equally from α_{2u}-globulin and appear to have undergone concerted evolution. The group 1 genes have more silent base substitutions relative to α_{2u}-globulin suggesting conservation of protein function by selection.

There are approximately 24 MUP genes which are members of groups 1 or 2. The other 6 to 11 genes are members of groups 3 or 4 (Al-Shawi *et al.*, 1989). They are also found near the *Mup-1* locus but may not be arranged in 45kb palindromes (Shi *et al.*, 1989). Two group 3 and three group 4 genes have been identified (Ma 1987; Shahan *et al.*, 1987b). Groups 3 and 4 are more closely related to each other than to groups 1 or 2. Group 3 genes are further distinguished because they code for the glycosylated MUPs (Kuhn *et al.*, 1984; Ma 1987). Groups 3 and 4 differ from the group 1 genes in 15% of nucleotides in the coding region (Kuhn *et al.*, 1984, Ma, 1987) and 22% of amino-acids. A similar subgroup of approximately six α_{2u}-globulin genes differ from the others by 5% of nucleotides clustered in exon 3 (13% difference) (Laperche *et al.*, 1983; Gao *et al.*, 1989).

Although the 35 MUP genes are highly homologous one region shows large variation. This is approximately 50 nucleotides upstream of the cap site, where there is a stretch of adenine residues or adenine

interspersed with cytosine which varies in length from 65 nt (group 1 gene, BS1) to 13nt (group 1 gene, BL6-51) Al-Shawi *et al.*, 1989; Held *et al.*, 1987. The α_{2u}-globulin genes have a similar hypervariable region in the same position.

EXPRESSION OF MUP AND α_{2u}-GLOBULIN

Splicing

MUP genes have seven exons and number of different patterns of splicing occur. Two major sizes of MUP mRNA (0.77 and 0.9kb) detected in the liver are produced by different splicing in the 3' non-coding region. The long message contains a short version of exon 6 called exon 6a and exon 7. The short message contains a long version of exon 6 and no exon 7. A second type of long mRNA contains an extra thirty-one nucleotides from the 5' end of intron 6 before the splice with exon 7 (Shahan *et al.*, 1987a, Chave-cox 1986). The rat liver α_{2u}-globulins are spliced like the long mRNA without the extra thirty-one nucleotides. Several different α_{2u}-globulin mRNAs are present in the rat submaxillary gland. They are made from two different genes using a combination of six different splice acceptor sites in exon 6, intron 6 and exon 7 and two different polyadenylation signals. None of the splice acceptor sites is the same as that used by liver α_{2u}-globulin mRNAs (Gao *et al.*, 1989). The reason for the different patterns of splicing and their effects on mRNA stability are not known. In some cases sequences present in introns have been shown to be important for mRNA stability and translational efficiency it is not known whether any important sequences are present in intron 6 of MUPs or α_{2u}-globulins.

Pseudogenes

The group 2 pseudogenes are either not expressed or expressed at very low levels. There are several features of the group 2 genes which would be detrimental to expression (Clark *et al.*, 1985) and no group 2 RNA was detected in liver by hybridization of group 2 specific oligonucleotides (Shahan *et al.*, 1987a; McIntosh and Bishop 1989). Whittaker (1989) showed that in transgenic mice the group 2 promoter was able to direct a very low level of expression of the Herpes Simplex virus thymidine kinase reporter in the liver (5% of group 1 promoter).

Functional genes

Tissue-specificity

Members of the four groups of MUP genes defined by sequence homologies also have similar patterns of expression. Many group 1 MUPs are expressed in the liver and a subset of group 1 MUPs are expressed in the mammary gland and perhaps the sebaceous glands (Held *et al.*, 1989). Group 3 MUPs are expressed in the liver. Different closely related subsets of the group 4 MUPs are expressed in the submaxillary gland (Hastie *et al.*, 1979), lachrymal gland, parotid gland and sublingual gland (Shaw *et al.*, 1983; Shahan *et al.*, 1987a). In addition in each tissue the level, timing and hormonal regulation of expression is different. The expression of α_{2u}-globulins is similar to MUPs in that subsets of α_{2u}-globulins are expressed in liver, submaxillary (Antakly *et al.*, 1982, Laperche *et al.*, 1983, Gubits *et al.*, 1984) and lachrymal glands (Gubits *et al.*, 1984). However in contrast to MUPs, all of the α_{2u}-globulin genes are expressed in the preputial and mammary glands (Held and Gallagher, 1985).

The level of MUP mRNA in each tissue varies. The liver is the site if highest expression where MUP makes up 5% of the mRNA. The liver is also an important site of α_{2u}-globulin expression, however the highest levels of α_{2u}-globulin mRNA are found in the preputial gland. The levels of MUP and α_{2u}-globulin mRNA in other tissues is much lower than the liver ranging from 0.21% of total mRNA in the submaxillary gland to 0.02% of total mRNA in the sublingual gland (Shaw *et al.*, 1983).

Silent genes

Not all of the functional MUP genes are expressed. Two group 4 MUP genes, *Mup-1.5a* and *Mup-1.5b*, are each present in one copy in the genome of the inbred mouse strain Balb/cByJ (Shi *et al.*, 1989). Both genes are capable of making mRNA but *Mup-1.5b* is silent. The two genes are very closely related and differ by three nucleotides out of 5kb including 500 bases of 5' and 3' region and 4kb of transcription unit.

In the two inbred mouse strains Balb/c and C57BL/6 there are approximately twelve group 1 MUP genes but only six major group 1 proteins are made in the liver (Shaw *et al.*, 1983, Clissold *et al.*, 1984; Kuhn *et al.*, 1984). This means either that at least one protein is coded for by more than one gene or that up to half the group 1 genes are silent. In two cases two group 1 genes were found to code for the same protein (Held *et al.*, 1987), however in Balb/cJPt null mice deletion of approximately half of the group 1 genes has led to the loss almost all group 1 expression in the liver. This is consistent with half of the group 1 genes being comparatively silent (Shi *et al.*, 1989, Duncan *et al.*, 1988). Also consistent with this is the fact that only six different group 1 MUP cDNA clones have been described, five from Balb/c and one from C57BL/6 (Kuhn *et al.*, 1984; Shahan *et al.*, 1987b; Chave-

Cox, 1986). Further studies may lead to the discovery of silent group 1 genes similar to the silent *mup1.5b* gene.

Timing of expression

The timing of expression varies between different MUP and α_{2u}-globulin genes. Expression of MUP in the submaxillary gland begins at approximately one week of age, reaches maximum expression at three weeks of age and drops again in adults (Shaw *et al.*, 1983). α_{2u}-globulins show a similar pattern of expression to MUPs in the submaxillary gland. Expression of α_{2u}-globulin begins at ten days, reaches a maximum at thirty five days and decreases in the adult (McInnes *et al.*, 1986). Expression of the lachrymal MUP gene is equal in young and adult mice (Shaw *et al.*, 1983). α_{2u}-globulin expression is different to that of MUP in the lachrymal gland. It begins at 15 days and increases to a maximum in the adult (McInnes *et al.*, 1986). In the mammary gland, MUP is expressed during pregnancy and stops at parturition (Shaw *et al.*, 1983).

The onset of MUP expression in the liver coincides with the onset of sexual maturity. It begins at about 3 weeks after birth and increases to reach the adult level at 6 to 7 weeks (Barth *et al.*, 1982). α_{2u}-globulin expression also begins at the onset of sexual maturity. Transcription begins at 25 to 44 days after birth (Kulkarni *et al.*, 1985). Expression of MUP and α_{2u}-globulin in the liver is reduced in old rats and mice. This is associated with a loss of matrix association of α_{2u}-globulin genes (Murty *et al.*, 1988).

Hormonal regulation

In some tissues the expression of MUP is sexually dimorphic. The degree of sexual dimorphism varies in different mouse strains and species. Most studies have utilized the Balb/c and C57BL/6 inbred strains, and the following discussion relates to these strains.

The expression of the submaxillary MUP genes (*Mup-1.5a* and *Mup-1.5b* (Shi *et al.*, 1989) is not sexually dimorphic and they are expressed equally in males and females in both the submaxillary and the

sublingual glands (Shahan *et al.*, 1987a). The lachrymal MUP gene (*Mup-1.4*) is expressed five times more highly in male than female C57BL/6 mice at the mRNA level and is induced by testosterone in hypophysectomised mice or normal female mice (Shaw *et al.*, 1983). The expression of α_{2u}-globulin in the lachrymal gland is also sexually dimorphic with a male to female mRNA ratio of three to one (Gubits *et al.*, 1984). The level of expression of α_{2u}-globulin in the preputial gland is not sexually dimorphic.

Expression of both MUP and α_{2u}-globulin in the liver is sexually dimorphic. In C57BL/6 mice the mRNA level in females is approximately one fifth of that in males. Expression of α_{2u}-globulin is high in male rats whereas in female rats it is barely detectable (Kulkarni *et al.*, 1985). The loss of MUP or α_{2u}-globulin expression in a number of hormonally deficient mice as shown in Table I.4 implies that the expression of MUP and α_{2u}-globulin is dependent on steroids, thyroxine, insulin and GH.

Some hormones can affect the action of other hormones and it is often difficult to distinguish between a direct effect of the hormone or an indirect effect through another hormone. For example, thyroxine and

Table I.4 Expression of MUP and α_{2u}-globulins in hormonally deficient mice and rats

	% ADULT MALES	
	MICE	RATS
ADULT FEMALES	20	0
TFM MALES (NO ANDROGEN RECEPTOR)	3	NA
CASTRATED MALES	ND	15
THYROIDECTOMIZED MALES (NO THYROXINE)	2	ND
HYPOTHYROID MALE	NA	10
lit/lit MALES (LOW GH)	0.7	NA
HYPOPHYSECTOMIZED MALES (NO PITUITARY HORMONES)	0.2	0
ADRENALECTOMIZED MALES (NO GLUCOCORTICOIDS)	ND	23
DIABETIC MALES (NO INSULIN)	NA	20

NA - Not applicable ND - Not determined

glucocorticoids enhance GH gene expression, synthesis and release (Martinoli and Pelletier 1989). Thyroxine also enhances GH receptor expression in the liver (Hochberg *et al.*, 1990) and insulin affects the level of thyroxine and GH receptors in the liver (Murty *et al.*, 1986). Recent studies have been directed at determining which hormone has a

direct effect on MUP and α_{2u}-globulin expression.

Some hormonally deficient rats and mice result from genetic lesions. Hormonal deficiency can also be induced by surgical, chemical or (more recently) transgenic ablation (Borrelli *et al.*, 1989, Behringer *et al.*, 1988). Administration of hormones to hormonally deficient mice or rats which lack MUP or α_{2u}-globulin expression can restore MUP or α_{2u}-globulin mRNA levels. Sometimes expression can be induced by administration of a hormone other than the one missing due to the primary lesion. In this way the hormone which has the most immediate effect can be identified.

The following experiments show that androgens can be fully replaced by a combination of GH and thyroxine. 100% expression is taken as the normal MUP mRNA levels in liver. Testicular feminized mice are genetic males with a lesion in the androgen receptor which are therefore unable to respond to androgens making them phenotypically female. Administration of a combination of GH and thyroxine can completely replace the need for androgens to achieve 100% MUP expression in the liver (Knopf *et al.*, 1983). On the other hand if hypophysectomized female mice which lack all the pituitary hormones including GH and TSH are given testosterone they do not show increased MUP expression over basal level or that induced by a combination of GH and thyroxine (Knopf *et al.*, 1983). Therefore androgens cannot act to increase MUP expression in the absence of a functional hypopituitary system, whereas GH and thyroxine can. Thyroidectomized female mice given testosterone show 20% MUP expression whereas intact female mice given testosterone show 100% expression (Clissold *et al.*, 1984) which indicates that as well as a functional hypopituitary system, thyroxine is required for the response to androgens. These results show that after thyroidectomy, hypophysectomy or in mice with androgen receptor deficiencies, androgens

cannot restore MUP mRNA levels whereas a combination of GH and thyroxine can.

It is thought that GH is responsible for the sexual dimorphism of MUP expression.Plasma GH levels are sexually dimorphic as discussed previously. Female mice have a high basal level which is composed of a series of small but frequent peaks. Males have a low basal level which is almost undetectable, and spikes of GH every 3 to 3.5 hours. GH can be administered to mimic one or other of these patterns. Injection of GH every 12 hours is thought to imitate the male pattern and infusion of GH by minipump is thought to imitate the female pattern. GH administered by injection is more effective at inducing MUP mRNA than GH administered by infusion. *Lit/lit* mice are mutant dwarf mice which lack GH but are able to respond to exogenous GH administration. *Lit/lit* mice given GH injections had 100% MUP expression. Those given infusions had 30%, which is near to the female level of MUP expression. This indicates that the pattern of plasma GH can influence the sexual dimorphism of MUP expression (Norstedt and Palmiter, 1984).

The interactions between GH and thyroxine administered to hormone deficient rats and mice are described in table I.5. (Knopf *et al.*, 1983, Lynch *et al.*, 1982, Murty *et al.*, 1986). These results show that neither GH nor thyroxine alone can fully induce MUP or α_{2u}-globulin mRNA. In thyroidectomized mice both thyroxine and GH levels are depleted because thyroxine is important for GH transcription as discussed. Some effects of thyroxine on MUP and α_{2u}-globulin expression are due its effect on the GH gene but the inability of GH administration to induce MUP expression in the absence of thyroxine indicates that thyroxine is not only acting in this way but also affects the expression of MUP and α_{2u}-globulin more directly. It has been suggested that thyroxine affects the production of bound rather than unbound

ribosomes (Chatterjee *et al.*, 1983) and therefore favors the production of secreted rather than non secreted proteins in the liver.

A further complication of MUP expression in the liver is that different MUP genes respond to different extents to each hormone. Hormones could in principle change the level of MUP mRNA in the liver in either of two ways; by changing the transcription of all MUP genes equally or by specifically changing the transcription of one MUP gene. The actual situation is probably intermediate between the two extremes. Each hormone has some effect on all MUP genes but has a greater effect on one or more specific MUP genes. In the experiments referred to above the levels of urinary MUP and liver MUP mRNA were measured but individual MUPs were not examined. This may give a distorted view of the effect of hormones on MUP expression. Seven MUPs are produced in the liver of male C57BL/6 mice. The major protein products of group 1 genes are designated MUPs 1 to 5 (Knopf *et al.*, 1983), group 3 MUPs are designated MUP 6 and the origin of MUP 7 is not known. MUPs 2, 3 and 4 are present in higher levels than 1, 5, 6 and 7. In female C57BL/6 mice the major protein product is MUP 3. In thyroidectomized female mice given thyroxine, the levels of MUPs 1 and 5 were abnormally elevated. When thyroidectomized female mice were given testosterone the level of MUP 6 was elevated. When they were given GH by injection, MUP 3 was elevated to produce a pattern of expression which is similar to that of normal female mice (Knopf *et al.*, 1983). This suggests that lack of thyroxine prevents the induction of the male pattern of gene expression by injection of GH.

The group 3 MUPs, in the liver, are regulated differently by hormones to the group 1 MUPs. The group 3 MUPs are relatively insensitive to thyroxine and show a smaller response to GH than group 1 MUPs (Kuhn *et al.*, 1984). Studying individual genes in the liver allows differences

Table I.5 The effect of GH and thyroxine administration on MUP and α_{2u}-globulin expression in hormonally deficient mice and rats

MICE (% ADULT MALES)		RATS (% ADULT MALES)	
HPX FEMALE	0.2	HPX MALE	0
HPX +bGH	30	HPX + bGH (1 injection)	8
HPX +THYROXINE	30	HPX + bGH (12 injections)	78
HPX +THYROXINE +bGH	300	HPX + THYROXINE + GLUCOCORTICOIDS	4
MICE (% ADULT MALES)		RATS (% ADULT MALES)	
THYROIDECTOMIZED FEMALE (THX)	2	HYPOTHYROID (HPT)	10
THX + bGH	8	HPT + bGH	89
THX + THYROXINE	60	HPT + THYROXINE	94
THYROID ABLATED FEMALE (TA)	0		
TA + bGH	0		
TA + THYROXINE	100		

in their hormone response to be analysed. The MUP genes are so highly homologous that the sequence changes which mediate different hormonal

regulation should be easily identified. Studies of other groups of closely related genes have yielded information about sequences involved in gene regulation. For example the DNA sequences of the α_1 antitrypsin genes of two mouse species differ by only 4% of nucleotides but they show quite different usage of enhancers and promoters to drive transcription (Latimer *et al.*, 1990). Again, in the kallikrein genes single or double nucleotide differences in the regulatory elements are responsible for differences in the tissue specificity of expression (Wines *et al.*, 1989).

Genetic regulation

The genetic environment is known to influence the regulation of MUP expression. Previous studies have revealed variability in MUP patterns in different strains of mice (Szoka and Paigen 1979). Duncan *et al.*, (1988) analysed two Balb/c substrains with different urinary MUP patterns and observed two modulator loci. One was tightly linked to the MUP locus and the other segregated independently. Balb/cJPt MUP null mice had very low levels of group 1 proteins in their urine. The phenotype was linked to a variant *Mup-1* locus in which approximately half of the group 1 genes had been deleted (Shi *et al.*, 1989). The second phenotypic effect was due to a *trans*-acting modulator. The Balb/c substrain called Balb/cJPt MUP 4.1^{lo} showed reduced expression of a single group 1 protein (MUP 4.1). The expression of MUP 4.1 in these mice was highly variable whereas the overall level of MUP expression was not affected. The *trans*-acting modulator is probably located on chromosome 15.

Other variation in MUP proteins in urine was observed in recombinant inbred (RI) strains between C57BL/6 and DBA/2J. Some of these RI

strains showed a new MUP phenotype involving reduced expression of one group 1 protein called MUP 2.1. This was mediated in *trans*, but the genetic locus responsible for the effect was not located (Ma, 1987).

Interesting observations relating to MUP function were made by Sampsell and Held (1985) who analysed MUP expression in a number of wild derived mice species. Expression occurred in the male liver in all species but expression in the female liver varied from none at all to equality with the male. In one case variation in expression was shown to act in *cis* because MUP genes retained their high or low levels of expression in female F_1 hybrid mice. The relative expression of group 1 and group 3 like genes in the livers varied from species to species. In inbred mice 90% of liver MUP mRNA is group 1 and 10% is group 3. In some wild derived species almost all mRNA was group 1-like whereas others had a high proportion of group 3-like mRNA. Similarly, some species had more group 3-like genes and some had more group 1-like genes. Not all species had the very large numbers of MUP genes observed in inbred strains. These results show that features of MUPs in inbred strains, such as a large number of genes, sexual dimorphism of liver expression and a high level of group 1 expression are by no means universal amongst wild mouse species.

Intrastrain variation was described by McIntosh and Bishop (1989) who found that the level of hepatic MUP mRNA in different Balb/c females varied from 1 to 14% of the male level. This variation was specific to MUP mRNA and was not found in other liver mRNAs. As MUPs are hormonally regulated it was suggested that this was due to variation in the hormonal status of individual mice.

IDENTIFICATION OF SEQUENCES INVOLVED IN MUP AND α_{2u}-GLOBULIN EXPRESSION

Transcriptional regulation

The developmental timing and sexual dimorphism of MUP expression are regulated at the level of transcription. Higher levels of transcription are found in adult males (96 times higher) than in seven day old males. Higher transcription rates were found in adult males (7 times higher) compared with adult females (Derman, 1981). The transcription rate in females can be raised to the level seen in males by treatment with testosterone (Al-Shawi *et al.*,1988). Since testosterone can be replaced by a combination of pulsatile GH and thyroxine then the action of these hormones also occurs at the level of transcription. It is not known whether GH and thyroxine both act directly on hepatocytes and in particular whether there is a direct receptor mediated effect of thyroxine on MUP transcription. In isolated hepatocytes the transcription of MUP genes was not raised by treatment with GH or thyroxine (Speigelberg and Bishop, 1988). This may be because the pattern of GH administration is crucial to increase transcription of MUP genes.

Cell Culture

Within the liver, MUP genes are expressed only in differentiated hepatocytes. Expression of MUP genes is rapidly lost in isolated hepatocytes and hepatomas along with that of many other liver specific genes (Spiegelberg and Bishop, 1988; Held *et al.*, 1989). This means that it is not possible to study the regulation of MUP gene expression in these systems. Expression of α_{2u}-globulin is reduced during the acute phase and transcription of α_{2u}-globulin falls rapidly during the

first 24 hours after acute phase stimulation (Schreiber *et al.*, 1989). This further mode of regulation suggests that the expression of MUP and α_{2u}-globulin genes can easily be switched off by stress related factors.

Transgenic mice

The sequences which direct both liver-specific and sexually dimorphic expression of group 1 MUP genes reside in 2.2kb of 5' proximal sequences. Three constructs containing this promoter fragment directed expression of three different reporter genes in a liver specific manner and two also in a sexually dimorphic manner in transgenic mice (Held *et al.*, 1989; Al-Shawi *et al.*, 1988; Al-Shawi *et al.*, submitted). The sequences which direct liver specific and sexually dimorphic expression of α_{2u}-globulin are contained within a 7kb fragment containing 2.7kb of 5' proximal DNA and an α_{2u}-globulin structural gene (Costa Soares *et al.*, 1987). Similar regions probably direct tissue specific expression of all MUP genes since a 7kb fragment of the submaxillary gene *Mup1.5b* including 4.4kb of 5' proximal region contains all of the sequences necessary to direct expression to the submaxillary gland (Shi *et al.*, 1989)

The 7kb fragment of α_{2u}-globulin also contains sequences necessary for expression in the preputial gland and a low level of expression in the submaxillary and lachrymal gland. Expression of endogenous α_{2u}-globulin is normally found in these tissues. Similar sequences may occur in MUP genes because the 2.7kb proximal promoter fragment directs a low level of expression of reporter genes to the preputial gland (Held *et al.*, 1989; Al-Shawi *et al.*, 1988 and submitted). The preputial gland is not a normal site of endogenous MUP expression which suggests that these

elements are repressed in endogenous MUP genes.

It is not known which part of the 2.7kb 5' proximal promoter region contains the *cis*-acting sequences necessary for MUP expression. The 5' proximal 300bp alone cannot direct liver specific expression of hsvTK in transgenic mice, however studies *in vitro* using 300bp of α_{2u}-globulin showed that this region contains all sequences necessary for liver specific expression *in vitro*.

Nuclear Extracts

The promoters of two α_{2u}-globulin genes have been studied *in vitro*. In one case male-specific transcription of the α_{2u}-globulin gene was achieved whereas in the other case it was not (Sarkar and Feigelson 1989; Sierra, 1990). The nuclear extracts used in both cases were made using the same method and the differences between the two experiments was the particular α_{2u}-globulin gene studied and the presence or absence of the coding region. Male-specific transcription was achieved using the a construct containing part of the α_{2u}-globulin gene 207 containing 639bp of 5' sequence and 1395bp of coding region including intron 3 which is known to contain binding sites for nuclear factors in expressing tissues (Sarkar and Feigelson, 1989; Choy *et al.*, 1989). Male specific transcription was not achieved using 3kb of the 5' promoter region from an unidentified α_{2u}-globulin gene and a G-free cassette reporter (Sierra, 1990). This construct and a deletion containing 245bp of the 5' proximal region were able to drive liver specific expression of a G-free cassette. The 245bp region contained binding sites for 6 factors present in both liver and spleen nuclear extracts, some of which showed different binding in liver and spleen. Oligonucleotides containing binding sites for known *trans*-acting

factors in the albumin promoter were used in competition experiments to show that one of the factors was C/EBP and another was pseudo NF1, both of which are liver enriched.

AIMS OF THESIS

In general liver-specific, hormonal and developmental regulation of gene expression occurs when proteins bind in a sequence-specific manner to DNA. Small changes in the sequence of the DNA to which these proteins bind will affect their binding and thus the way in which a gene is regulated. The group 1 MUP genes are very closely related so that their sequences are almost identical yet some genes respond to hormones in different ways than others. By examining the differences in sequence of group 1 genes, changes in sequence which could potentially be responsible for causing the different types of hormonal regulation can be identified. In order to do this unique group 1 MUP genes must be identified and their regulation characterized. In practical terms this means that group 1 mRNAs or proteins must be uniquely distinguished.

Two methods were used to identify and distinguish group 1 MUP genes. Firstly, short oligonucleotides were designed which could distinguish between sequences which differed by one base pair and hence distinguish between closely related group 1 mRNAs. Secondly, group 1 proteins which differ by a single amino-acid were separated by IEF and the products of particular group 1 genes were identified by translating them *in vitro*. Oligonucleotides and IEF were then used to compare the regulation of group 1 MUP genes. The sexually dimorphic expression of allelic group 1 genes was compared in two inbred strains of mice. The induction of different subsets of group 1 MUP genes was compared in GH deficient

mice treated with different patterns of GH. Subsets of group 1 MUP genes were identified which are regulated differently and their regulation is discussed.

CHAPTER 1

SINGLE COPY GROUP 1 MUP GENES

All MUP genes map to a single locus on chromosome 4 (Bennett *et al.*, 1982; Bishop *et al.*, 1982) called *mup-1*. Most inbred strains of laboratory mice carry one of two principal alleles at this locus which are thought to derive from two alleles present in the wild population of *Mus. m. domesticus* (Hudson *et al.*, 1967; Duncan *et al.*, 1988). Balb/c mice are amongst those with the *mup-1*^{a} allele whereas C57BL/6 are amongst those with the *mup-1*^{b} allele and these strains are the most commonly used in studies of MUP genes and their regulation. Both have approximately thirty-five MUP genes which can be divided into four groups on the basis of sequence similarities (Al-Shawi *et al.*, 1989). The four groups, named groups 1, 2, 3 and 4, are of similar size in both strains. Groups 1 and 2 contain twelve to fifteen genes (Bishop *et al.*, 1982), groups 3 and 4 contain two to four genes (Shahan *et al.*, 1987; Kuhn *et al.*, 1984). The aim of this project is to identify single copy group 1 MUP genes and compare their expression in the two strains.

Thirty-two group 1 genomic clones have been isolated from Balb/c and C57BL/6 libraries and analysed by restriction site mapping (Held *et al.*, 1987; Al-Shawi, 1985). Although the maps are very similar, many clones or small groups of clones have characteristic restriction fragments which distinguish them from the others. By mapping group 1 genes in genomic DNA, some of these fragments can be shown to be present in one copy per haploid genome and the small group of clones must represent multiple cloning events of the same unique MUP gene (Al-Shawi *et al.*, 1989). When a characteristic fragment is present in more than one copy per genome the small group of clones may be derived from different genes, and it is quite likely that these genes have

nucleotide differences which were not detected by restriction mapping.

There is a region of considerable divergence starting fifty base pairs upstream of the transcription start site of each group 1 gene. This hypervariable region contains a stretch of A residues or A and C residues which varies in length between thirteen and sixty-two nucleotides. Sequence data for this region has been obtained from fourteen group 1 genomic clones isolated from either Balb/c or C57BL/6 (Al-Shawi *et al.*, 1989, Held *et al.*, 1987). Of the fourteen sequences, only two are identical in this region. This suggests that the hypervariable region is probably distinct in each group 1 MUP gene. The sequence of the hypervariable region is a useful method for comparing and distinguishing genomic clones which have identical restriction maps.

Two single-copy group 1 genes have been identified by restriction site mapping of genomic DNA in each of the two inbred strains. The four genes consist of two pairs of nearly identical genes which are referred to here as *Mup-1.1B*^{b}, *Mup-1.1B*^{a}, *Mup-1.1C*^{b} and *Mup-1.1C*^{a}. The two members of each pair are thought to be closely related by descent and each strain contains one member of each pair. Genomic and cDNA clones of these genes have been isolated (Al-Shawi, 1985; Kuhn *et al.*, 1984; Held *et al.*, 1987; see Table 1.1). Clones of *Mup-1.1B*^{a} have identical restriction maps but differ from clones of *Mup-1.1B*^{b} by one restriction site. The hypervariable region of a *Mup-1.1B*^{b} genomic clone, CL5 is different from that of a *Mup-1.1B*^{a} genomic clone, BS1 in the central part but identical in the flanking regions. This suggests that the two hypervariable regions are recent expansions or deletions of the same original sequence (Al-Shawi *et al.*, 1989).

All genomic clones of *Mup-1.1C*^{a} and *Mup-1.1C*^{b} have the same restriction

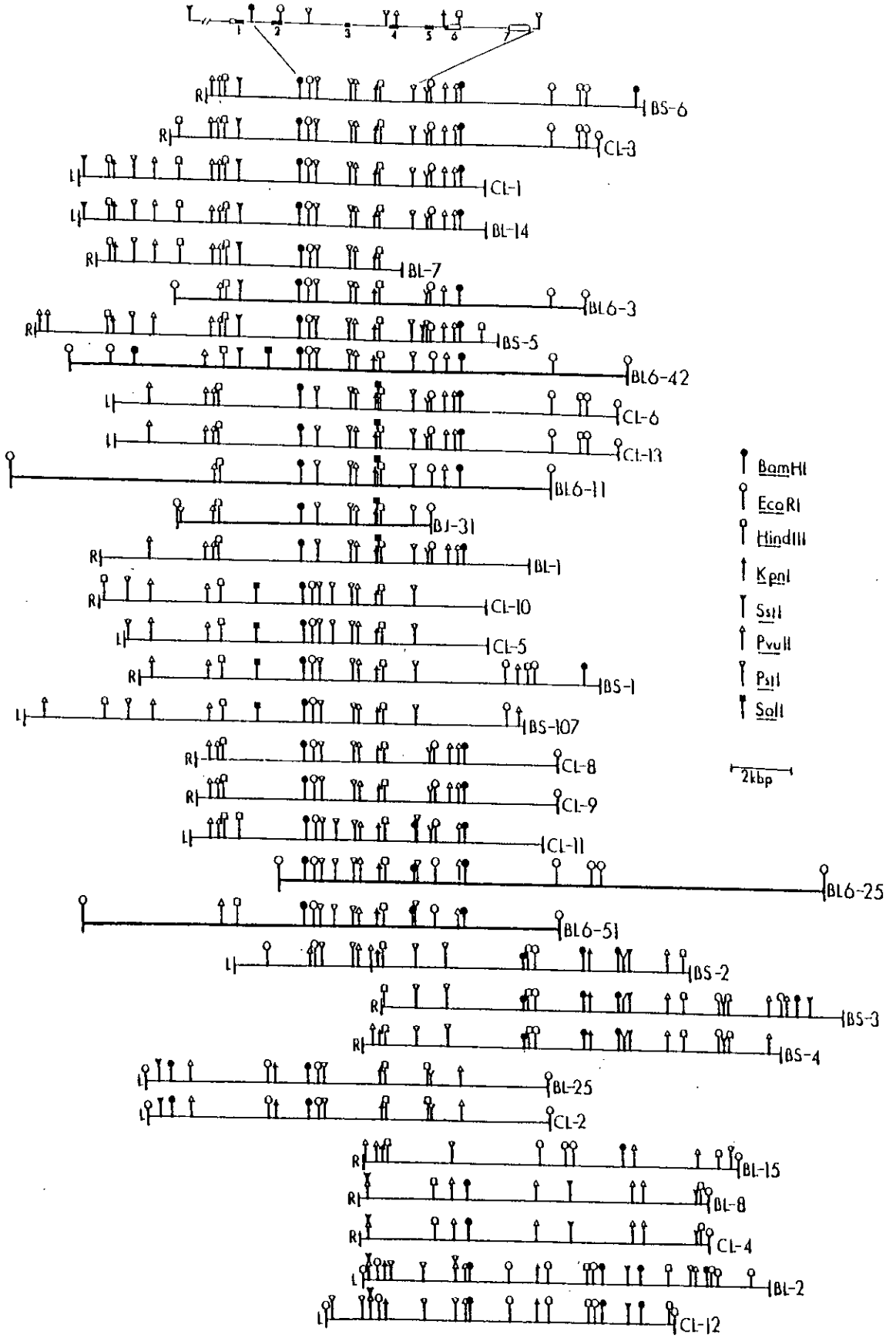
TABLE 1.1 Unique group 1 MUP genes

NAME OF GENE	UNIQUE RESTRICTION SITE DIFFERENCE	NAME OF STRAIN-SPECIFIC GENE	STRAIN	GENO-MIC CLONES	cDNA CLONES
Mup-1.1C	lack EcoRI site in exon 2	*Mup-1.1C*^{a}	Balb/c	BL1 BJ-31	cLiv6 MUP8
		Mup-1.1C^b	C57	CL6 CL13 BL6-11	p499
Mup-1.1B	Lack EcoRI site 3' to exon 7	*Mup-1.1B*^{a}	Balb/c	BS1 BS107	cLiv1
		Mup-1.1B^b	C57	CL5 CL10	-

map (Figure 1.1). The sequences of the hypervariable regions from two *Mup-1.1C*^{a} clones; BL1 and BJ-31 and one *Mup-1.1C*^{b} clone; BL6-11 have been determined. Each hypervariable region differs from the others by one nucleotide which is a very small difference compared with the additional 43 nucloetides in some group 1 genes (Held *et al.*, 1987; Al-Shawi *et al.*, 1989). The sequence similarity between group 1 genes in 800bp of the 5' proximal region is very high (5 to 10 nucleotide differences) (Held *et al.*, 1987). In the 5' proximal regions of BL1, BJ-31 and BL6-11 there are only three nucleotide differences which suggests a close relationship. BL1 and BJ-31 were derived from two

FIGURE 1.1

Restriction maps of MUP genomic clones in lambda vectors. Diagram extracted from Al-Shawi (1987, THESIS) with lambda clones from Held *et al.*, (1987) (BL6-3, BL6-42, BL6-11, BJ-31, BL6-25 and BL6-51) inserted in appropriate places. The clones have been mapped with the restriction enzymes shown. Clones from Held *et al.* (1987) were mapped using a cDNA clone as a probe which means that if there is more than one restriction site for an enzyme outside the mRNA region then the more distant restriction sites were not identified. The clones BS6 down to BL6-51 inclusive are clones of group 1 MUP genes. The clones BS-2 to BL-15 inclusive are clones of group 2 MUP genes. The clones BL-8 to CL-12 inclusive are clones of group 3 or 4 MUP genes. The clones BS-6, BL-14, BL-7, BS-5, BJ-31, BL-1, BS-1, BS-107, BS-2, BS-3, BS-4, BL-25, BL-15, BL-8 and BL-2 are clones isolated from Balb/c libraries. The other clones were isolated from C57BL/6 libraries.


1
2
3
4
5
6
7
BS-6
CL-3
CL-1
BL-14
BL-7
BL6-3
BS-5
BL6-42
CL-6
CL-13
BL6-11
BJ-31
BL-1
CL-10
CL-5
BS-1
BS-107
CL-8
CL-9
CL-11
BL6-25
BL6-51
BS-2
BS-3
BS-4
BL-25
CL-2
BL-15
BL-8
CL-4
BL-2
CL-12
BamHI
EcoRI
HindIII
KpnI
SstI
PvuII
PstI
SalI
2kbp

different Balb/c substrains which may account for two single nucleotide differences (one of which is in the hypervariable region) in the first 350bp of 5' proximal region of the two clones. There is a single nucleotide difference within the coding regions of *Mup-1.1C*^{b} and *Mup-1.1C*^{a} which can be used to distinguish between mRNA from these two genes.

Group 1 mRNAs can differ by only a single nucleotide change which means that very stringent techniques have to be used to distinguish mRNA from a particular MUP gene from all the other group 1 mRNA in the liver. Four techniques have been used to distinguish between very similar mRNAs; ribonuclease protection, hybridization of oligonucleotides, primer extension and the polymerase chain reaction (PCR). The use of these four techniques is described in Table 1.2.

Table 1.2 Methods of Distinguishing Closely Related RNAs

RIBONUCLEASE PROTECTION

The anti-sense RNA from a single cloned template is hybridized to the mixture of mRNA molecules. The RNA which is perfectly complimentary to the probe will protect it throughout its length. The probe hybridized to mismatched RNAs will be cleaved internally.

HYBRIDIZATION OF OLIGONUCLEOTIDES

Short oligonucleotides will form stable hybrids with perfectly matched targets, however, if there is a mismatch of a single nucleotide or more the hybrids will be unstable under stringent hybridization conditions.

PRIMER EXTENSION

The primer binds to a site adjacent to the sequence difference. The

extension is then carried out using a dideoxynucleotide or omitting a nucleotide in such a way that the reaction will terminate at a different point when the primer is bound to the desired target than if it is bound to the closely related sequences. The different length extension products can be distinguished by electrophoresis.

THE POLYMERASE CHAIN REACTION (PCR)

A small region of the closely related RNAs is amplified between two primers. The amplified fragment from the RNA of interest is distinguished from the others by restriction site polymorphisms. If the region amplified is short it is possible to use enzymes which cut frequently and many differences can be distinguished in this way.

Mup-1.1B, *Mup-1.1C*^{b} and *Mup-1.1C*^{a} mRNAs were distinguished by hybridization to three specific oligonucleotides. Two of the oligonucleotides were designed and used by McIntosh and Bishop (1989) to study the expression of *Mup-1.1C*^{a} and *Mup-1.1B*^{a} in male and female Balb/c liver. The probes were designed by comparing six group 1 coding sequences which were known. These consisted of five group 1 coding sequences from Balb/c including three cDNA clones (Kuhn *et al.*, 1984; Shahan *et al.*, 1987; Chave-Cox, 1986) and three genomic clones (Clark *et al.*, 1985) and one group 1 cDNA from C57BL/6 (Kuhn *et al.*, 1984). As there are approximately twelve highly homologous group 1 genes in each strain it is possible that there are unknown mRNAs which are identical to the probes. In order to test this the oligonucleotide probes were hybridized to genomic DNA and group 1 genomic clones derived from both Balb/c and C57BL/6.

RESULTS

The sequences of the three oligonucleotides are shown in Table 1.3. oBS1 and oBL1 are the two oligonucleotides designed by McIntosh and Bishop (1989). oBL1 does not hybridize to *Mup-1.1C*^{b} but this gene can be detected by a new oligonucleotide called oBL1RI. oBL1RI crosses the sequence in exon 2 of *Mup-1.1C*^{b} which is an EcoRI site in all MUP genes apart from *Mup-1.1C*^{b} and *Mup-1.1C*^{a}. oBL1RI is therefore specific for these two genes.

Table 1.3 Location of Oligonucleotides

OLIGO-NUCLEOTIDE	LEN-GTH	EX-ON	SEQUENCE*	GENE
oBL1RI	19	2	5'GAACTAAGGATTTCTCCAA	*Mup-1.1C*^{a} *Mup-1.1C*^{b}
oBL1	18	5	5'CACATAGTTGTGCAAACC	*Mup-1.1C*^{a}
oBS1	18	3	5'GATAATTCCGAGCACTCT	*Mup-1.1B*

* mismatch to other group 1 genes underlined

oBL1 and oBS1 were hybridized to a number of MUP genomic clones derived from Balb/c and C57BL/6 DNA (Table 1.4). oBL1RI was hybridized to the three clones which cannot be digested with EcoRI in exon 2 (Al-Shawi, 1985; Held *et al.*, 1987; Table 1.4). Maps of the four clones which

hybridized with oBS1 are shown in Figure 1.2. These clones are described later.

Table 1.4 Hybridization of oligonucleotides to MUP genomic clones

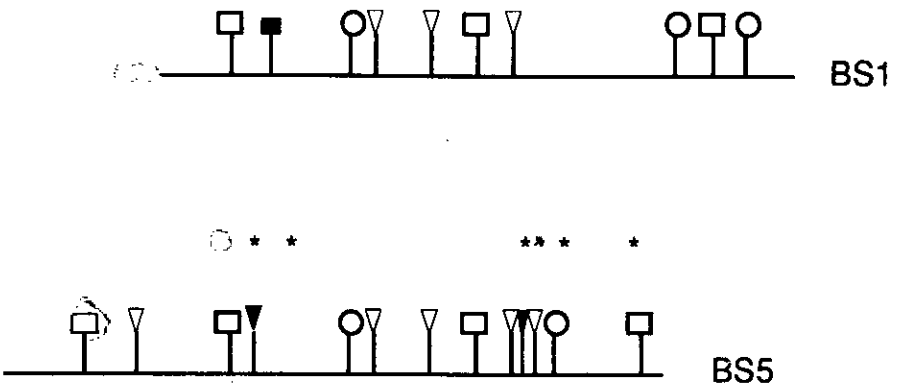
OLIGO NUCLEOTIDE	POSITIVE GENOMIC CLONES		NEGATIVE GENOMIC CLONES		CLONES NOT TESTED	
	Balb	C57	Balb	C57	Balb	C57
oBL1	BL1	-	BS6	CL3 CL6	BL14	CL1
			BS5	CL13	BJ31	BL6-3
			BS1	CL10	BS107	BL6-42
			BL7	CL5 CL8		BL6-11
				CL9		BL6-25
				CL11		
oBL1RI	BL1	CL6	ND	ND	ND	ND
		CL13				
oBS1	BS1	CL5	BS6	CL3 CL6	BL14	CL1
	BS5	CL10	BL7	CL13	BJ31	BL6-3
			BL1	CL8 CL9	BS107	BL6-42
				CL11		BL6-11
						BL6-25

ND - Not Determined

To estimate how many different genes in Balb/c or C57BL/6 could hybridize to oBL1, oBL1RI and oBS1 the oligonucleotides were hybridized to Southern blots of genomic DNA. The pattern of hybridization obtained was compared to the pattern obtained using a group 1 fragment probe

FIGURE 1.2

Restriction maps with five common six base pair cutters (shown) of genomic clones which hybridized to the oligonucleotide oBS1 (shown in table 1.2). Restriction site differences between BS-5, CL-5 or CL-10 and BS-1 are marked with an asterisk. There are six differences in BS-5, but only one between CL-5, CL-10 and BS1.


BS1
BS5
CL5
CL10
Eco RI
Hind III
Pst I
Sst I
Sal I

(G1F) which can hybridize to all group 1 genes. The G1F is a 700 base pair PstI restriction fragment from the genomic clone CL5 which includes the whole of exon 3. This probe was selected because it is expected to hybridize to the same restriction fragment of EcoRI, HindIII or PstI digested group 1 genes as the oligonucleotides.

In all of the genomic blots shown, restricted MUP genomic lambda clones were used as both size and copy number markers. The clones used in marker tracks were selected so that, where possible, fragments in the marker tracks would be the same size as those in genomic DNA. Figures 1.3a to 1.3d and 1.7 show the approximate sizes of restriction fragments used as markers in Figures 1.4, 1.5, 1.6 and 1.8. The copy number was determined by comparing the signal from the band in the copy number markers to the signal from the band in genomic DNA. The one copy marker contained 150pg of a 45kb lambda clone which was calculated to give the same signal as one copy per haploid genome in 10µg of genomic DNA.

The three genomic clones hybridized with oBL1RI were clones of *Mup-1.1C*^{a} or *Mup-1.1C*^{b} (Table 1.4, Table 1.1; Al-Shawi *et al.*, 1989; Held *et al.*, 1987) and the one which also hybridized with oBL1 was a clone of *Mup-1.1C*^{a} (Table 1.4; Table 1.1). The copy number of these genes was estimated by hybridizing oBL1 and oBL1RI to genomic DNA. Genomic DNA from Balb/c and C57BL/6 was restricted with HindIII or HindIII and EcoRI and hybridized to the G1F probe or the oligonucleotide oBL1 (Figure 1.4). An identical blot was hybridized to the oligonucleotide oBL1RI (data not shown.) It was previously shown that a 5.5 kb HindIII fragment is conserved in most group 1 genes of both strains (Al-Shawi, 1985). In addition, the EcoRI site in exon 2 mentioned previously allows the HindIII fragment from all group 1 genes except *Mup-1.1C*^{b} and *Mup-1.1C*^{a} to be cut by EcoRI into two fragments of 3 kb and 2.5 kb. In

FIGURE 1.3

Sizes of restriction fragments detected by the G1F probe in genomic clones used as markers in Figures 1.4, 1.5 and 1.6. The genomic clone digested to produce each fragment and the region which hybridizes to the G1F probe are indicated.

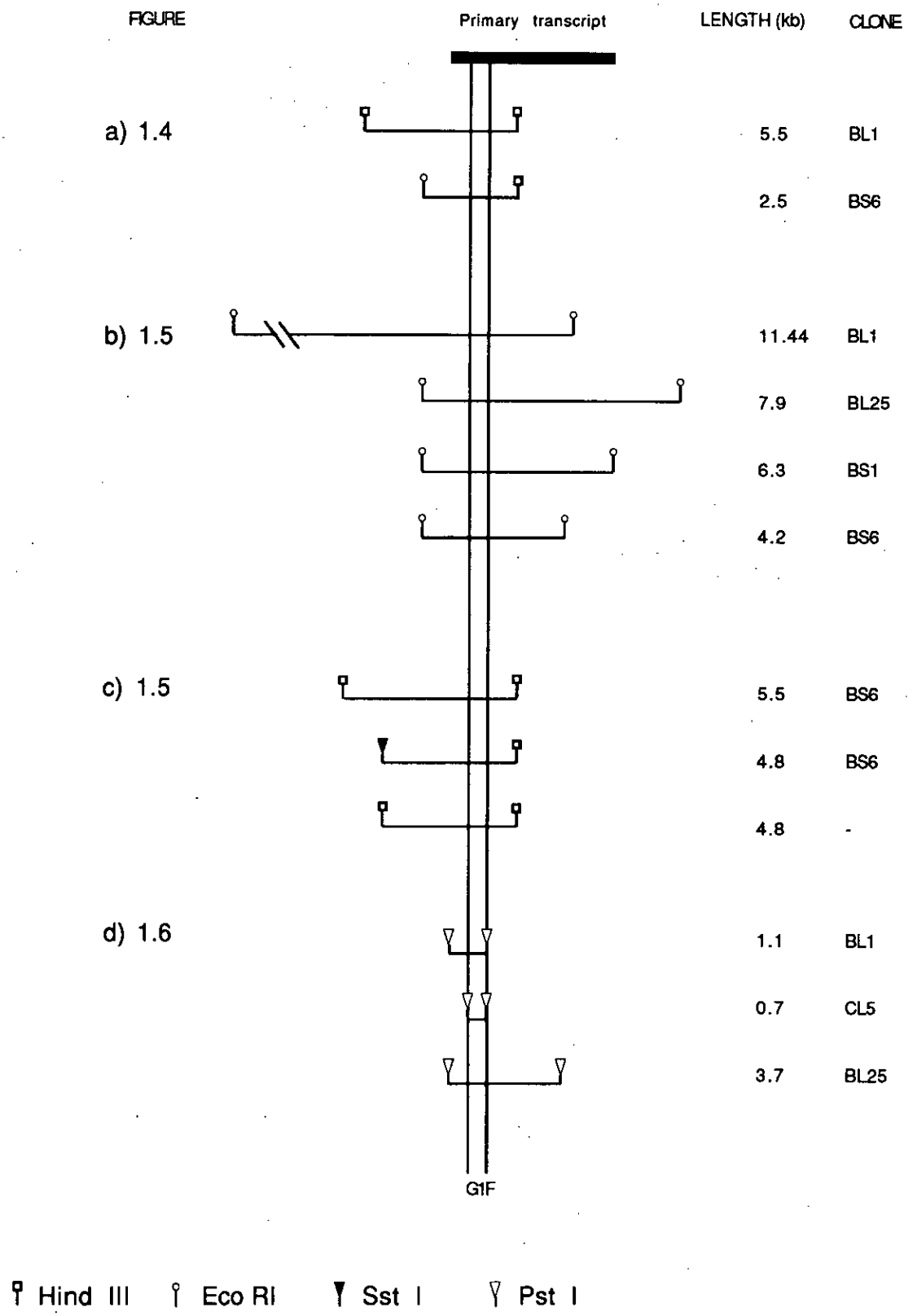


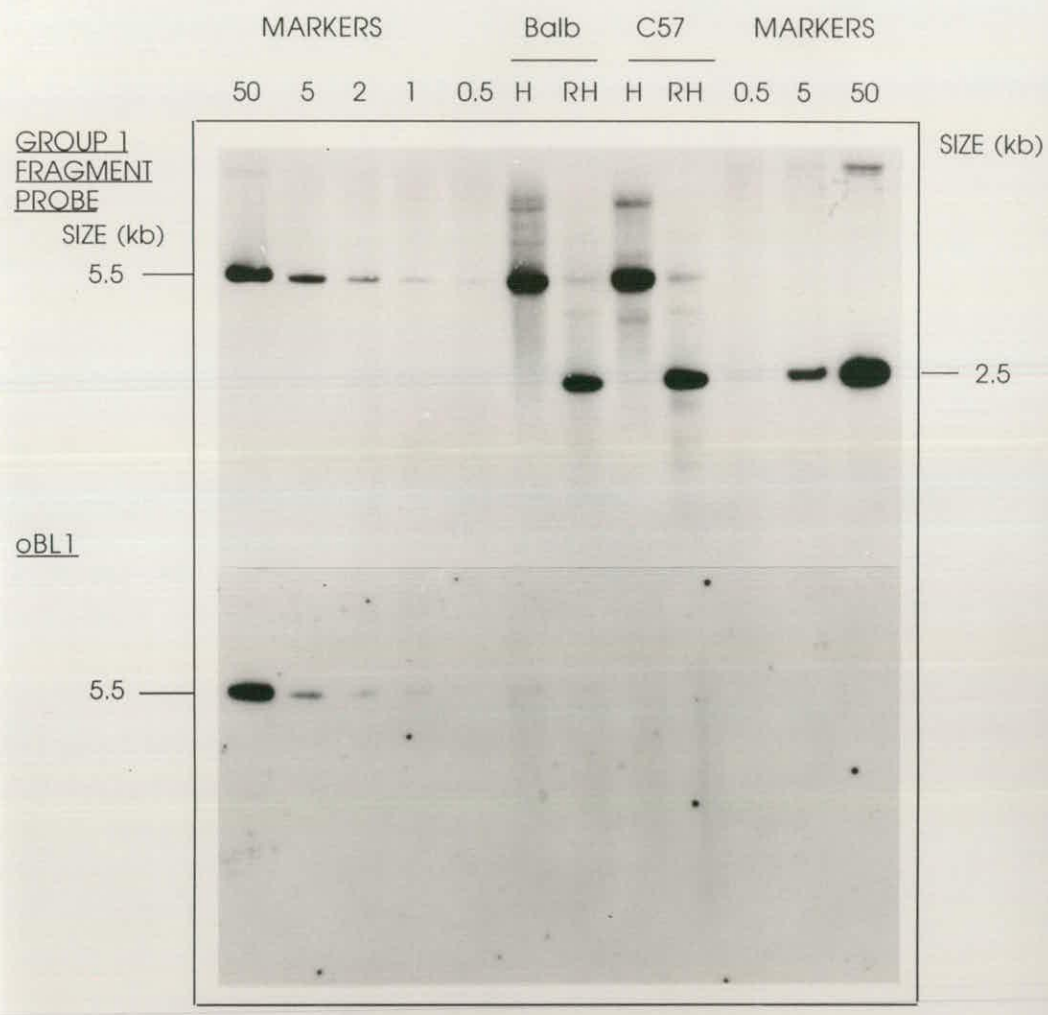
FIGURE
Primary transcript
LENGTH (kb)
CLONE
a) 1.4
5.5
BL1
2.5
BS6
b) 1.5
11.44
BL1
7.9
BL25
6.3
BS1
4.2
BS6
c) 1.5
5.5
BS6
4.8
BS6
4.8
-
d) 1.6
1.1
BL1
0.7
CL5
3.7
BL25
G1F
Hind III
Eco RI
Sst I
Pst I

FIGURE 1.4

A Southern blot showing the number of genes per genome of Balb/c and C57BL/6 mice which hybridized to the oligonucleotide oBL1.

The Southern blot contained 10µg of Balb/c or C57BL/6 genomic DNA per track cut with HindIII (H) or HindIII plus EcoRI (RH). The DNA fragments used as markers are shown in Figure 1.3a. On the left hand side marker tracks contained the equivalent of 50, 5, 2, 1 and 0.5 copies and on the right hand side, the equivalent of 0.5, 5 and 50 copies. Each marker track also continued 10µg of salmon sperm carrier DNA. The gel was 0.7% agarose.

The filter was hybridized twice, firstly to the oligonucleotide oBL1 (bottom panel) and then to the G1F probe (top panel). The autoradiographs were exposed for 1 hour at room temperature (top panel) or 3 weeks at -70°C with screens (bottom panel).


MARKERS
Balb
C57
MARKERS
50 5 2 1 0.5 H RH H RH 0.5 5 50
GROUP 1
FRAGMENT
PROBE
SIZE (kb)
5.5
SIZE (kb)
2.5
oBL1
5.5

a double digest with EcoRI plus HindIII, the 5.5 kb HindIII fragment from *Mup-1.1C*^{b} and *Mup-1.1C*^{a} is left uncut which allows these genes to be distinguished from the others. The 2.5 kb and the 5.5 kb fragments hybridize to the G1F probe. DNA from each strain contains a unique *Mup-1.1C* gene (Figure 1.4). The 5.5 kb HindIII fragment of this gene which is not cut with EcoRI is present at the same level as the 1 copy marker. The lower panel shows that only the 5.5 kb band derived from *Mup-1.1C* hybridizes to the oligonucleotide oBL1 and that oBL1 hybridizes to DNA from Balb/c not DNA from C57BL/6, thus oBL1 hybridizes with *Mup-1.1C*^{a} but not *Mup-1.1C*^{b}. The oligonucleotide oBL1RI hybridized to the 5.5 kb band derived from *Mup-1.1C* in both strains but also hybridized to other bands of non-MUP origin (data not shown). Thus oBL1RI hybridizes with *Mup-1.1C*^{a} and *Mup-1.1C*^{b}. This confirms that there is one copy of *Mup-1.1C* in the genome of each strain and also shows that *Mup-1.1C*^{a} is different to *Mup-1.1C*^{b}.

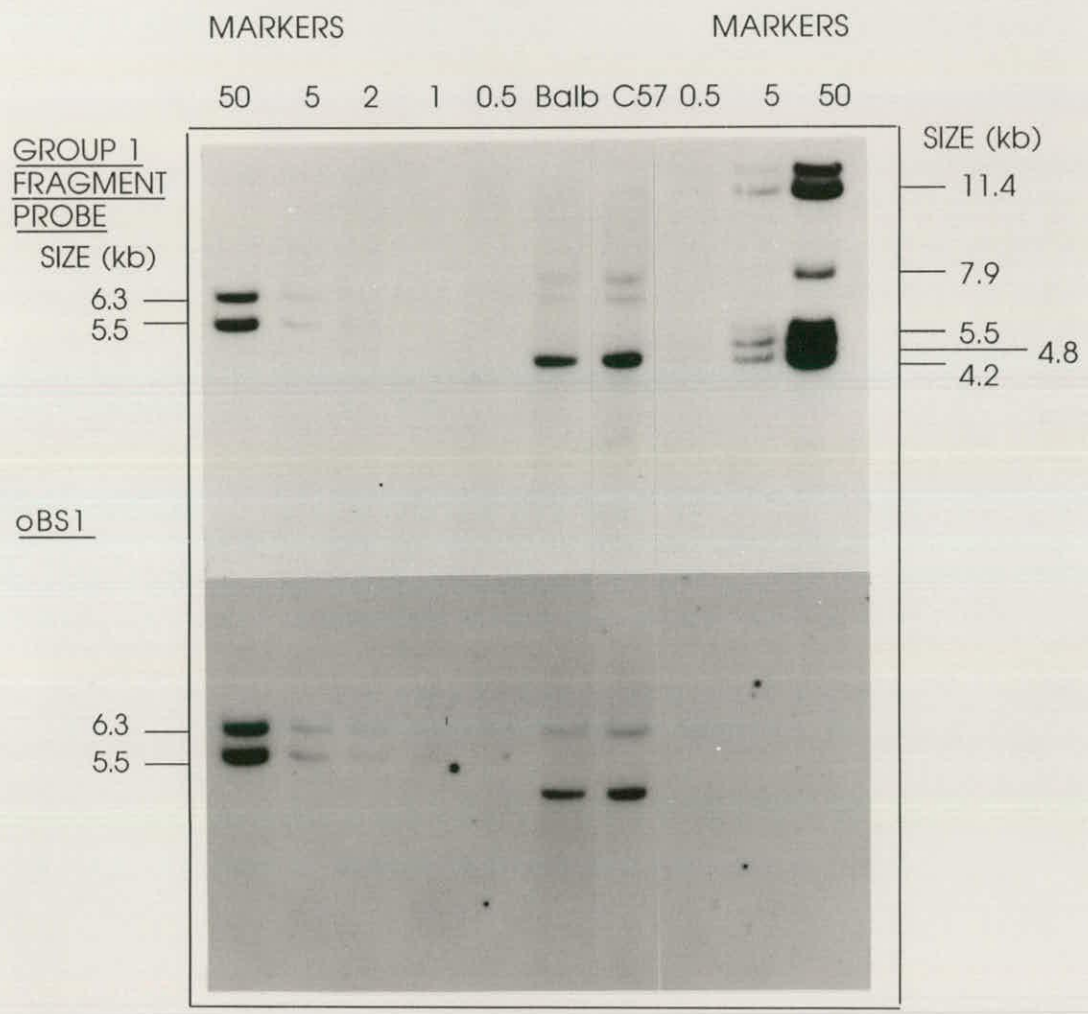
oBS1 hybridized with clones of *Mup-1.1B*^{a} (BS1) and *Mup-1.1B*^{b} (CL5 and CL10), however, it also hybridized with another clone derived from Balb/c which was not a clone of *Mup-1.1B*^{a} (Figure 1.2; Table 1.4; Table 1.1). Thus oBS1 is not specific to *Mup-1.1B*^{a}. In order to determine the number of genes in each strain which could hybridize to oBS1 restricted genomic DNA was hybridized with oBS1 and the G1F probe. By hybridization to the G1F probe DNA from *Mup-1.1B* can be distinguished from DNA from all other group 1 genes when cut with EcoRI (Table 1.1). Similarly, *Mup-1.1B*^{b} can be distinguished from *Mup-1.1B*^{a} when cut with PstI (Figure 1.2).

When MUP genomic clones are digested with EcoRI, four different fragments are produced which can hybridize to the G1F probe (Figure 1.3b). In EcoRI digested DNA from both strains, the G1F probe hybridized to four fragments (Figure 1.5). The 4.2kb, 6.3kb and 7.9kb

FIGURE 1.5

A Southern blot of EcoRI digested genomic DNA hybridized with the oligonucleotide oBS1. Tracks contained 10 µg of EcoRI digested Balb/c or C57BL/6 genomic DNA, or markers. The fragments present in marker tracks are shown in Figure 1.3b and c. On the left hand side marker tracks contained the equivalent of 50, 5, 2, 1 and 0.5 copies and on the right hand side the equivalent of 0.5, 5 and 50 copies. The gel was 0.6% agarose.

The filter was hybridized twice, firstly to the oligonucleotide oBS1 (bottom panel) and then to the G1F probe (top panel). The filters were exposed for 1 hour at room temperature (top panel) or 3 weeks at -70°C with screens (bottom panel).


MARKERS
MARKERS
50 5 2 1 0.5 Balb C57 0.5 5 50
GROUP 1 FRAGMENT PROBE
SIZE (kb)
6.3
5.5
oBS1
6.3
5.5
SIZE (kb)
11.4
7.9
5.5
4.8
4.2

fragments are the same size as restriction fragments from MUP genomic clones. The fragment which is >12kb is probably derived from *Mup-1.1C* (Duncan *et al.*, 1988; Shahan *et al.*, 1987b). The G1F probe hybridized more strongly to the 4.2kb fragment than the other EcoRI fragments, which means that this fragment is derived from more genes than the others.

The oligonucleotide oBS1 hybridized to the 4.2kb and 6.3kb fragments in genomic DNA from both strains (Figure 1.5) but not to the 7.9kb or >12kb fragments. The 4.2kb and 6.3kb fragments are the same size as those derived from the clones BS5 (4.2kb) and BS1 (6.3kb) which hybridize to oBS1 (Figure 1.5). Previous work by Al-Shawi *et al.*,(1989) suggested that *Mup-1.1B* is the only gene which contributes a 6.3kb fragment (Table 1.1). The above result shows that *Mup-1.1B* hybridized oBS1 in both strains. The 4.2kb fragment is contributed by the remaining group 1 genes except *Mup-1.1C* whereas the 7.9kb fragment is probably contributed by group 2 genes (Figure 1.1). Several group 1 clones do not hybridize to oBS1 (Tables 1.1 and 1.4). A comparison of the intensity of the hybridization signal using the oBS1 probe to that using the G1F probe reveals that over half of the group 1 genes which contribute to the 4.2kb band hybridize to oBS1. Hence, rather than oBS1 being specific to *Mup-1.1B*, it can in fact hybridize with over half of the group 1 genes in both strains of mice.

Two further mapping experiments were carried out to determine whether there were any differences between the Balb/c and C57BL/6 genes which were able to hybridize with oBS1. The genes were mapped with PstI and MspI. The two *Mup-1.1B*^{b} clones (CL5 and CL10) differ from the *Mup-1.1B*^{a} clone (BS1) in these digests (Figure 1.2).

When MUP genomic clones are digested with PstI, three fragments of 0.7,

1.1 and 3.7kb are produced which can hybridize with the G1F probe (Figure 1.3d). Five genomic clones isolated from C57BL/6 DNA produce the 0.7kb band which is not produced from any of the clones from Balb/c. Thus the 0.7kb fragment may be detected with the G1F probe in PstI digested genomic DNA from C57BL/6 but not Balb/c. Differences in the levels of hybridization to the 0.7kb fragment was indeed observed (Figure 1.6). Using the G1F probe, the 0.7kb PstI fragment was detected in Balb/c DNA at a very low level of less than one copy per genome. The 0.7kb PstI fragment was detected at a level equivalent to about 2 copies in C57BL/6 DNA. This difference between Balb/c and C57BL/6 DNA is also detected using the oligonucleotide oBS1. This suggests that two to three group 1 genes in the C57BL/6 genome contribute the 0.7kb Pst I fragment and at least one of them can hybridize with oBS1. No group 1 genes in Balb/c contribute a 0.7kb PstI fragment, but the weak hybridization of the G1F probe to this fragment means that it may be derived from a group 2, 3 or 4 gene in this strain. The other group 1 genes contribute to the 1.1kb PstI fragment and approximately half of them hybridize to oBS1. This indicates that there is a difference between the group 1 genes in Balb/c and C57BL/6 and the oligonucleotide oBS1 hybridizes to different genes in the two strains.

In previous mapping studies on group 1 MUP genomic clones, Al-Shawi (1985) found that several group 1 genomic clones had different MspI fragments. These are shown in Figure 1.7. An attempt was made to distinguish group 1 genes which hybridized with the oligonucleotide oBS1 from those which did not by digesting genomic DNA with MspI. The MspI fragments present in genomic clones used as markers in Figure 1.8 are shown in the upper part of Figure 1.7. They represent ten different MspI fragments derived from six different group 1 MUP clones. The G1F probe overlapped the MspI site in intron 2 and hybridized with two MspI fragments in some genes and clones. In Figure 1.8 the C57BL/6 genomic

FIGURE 1.6

A Southern blot of Pst I digested genomic DNA hybridized with the oligonucleotide oBS1. Tracks contained 10µg of Pst I restricted Balb/c or C57BL/6 genomic DNA or marker DNA. The fragments present in marker tracks are shown in Figure 1.3d. On the left hand side marker tracks contained the equivalent of 50, 5, 2, 1 and 0.5 copies and on the right hand side the equivalent of 0.5, 1, 2, 5 and 50 copies. The gel was 2% agarose.

The filter was hybridized twice, firstly to the oligonucleotide oBS1 (bottom panel) and then to the G1F probe (top panel). The filters were exposed for 1 hour at room temperature (top panel) or 3 weeks at -70°C with screens (bottom panel).

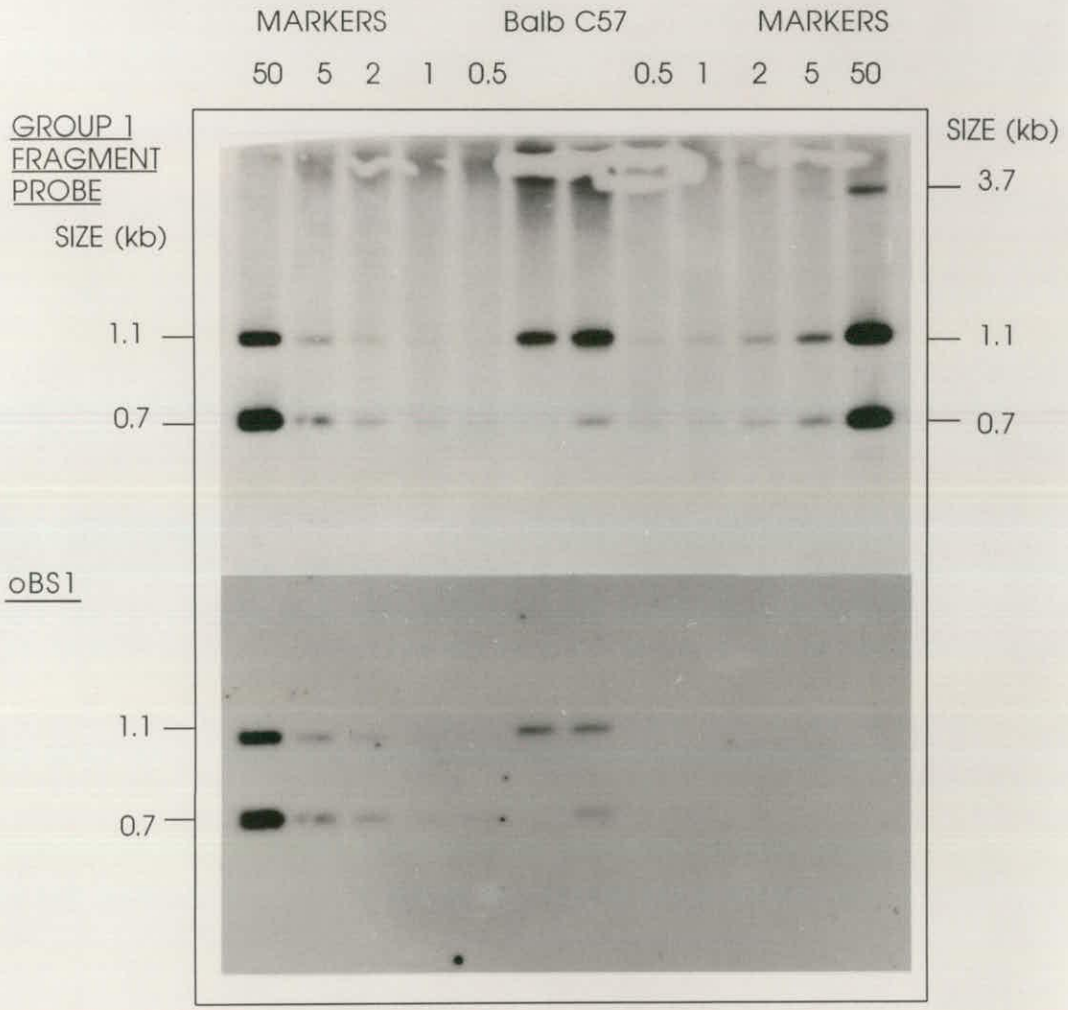

MARKERS
Balb C57
MARKERS
50 5 2 1 0.5
0.5 1 2 5 50
GROUP 1
FRAGMENT
PROBE
SIZE (kb)
1.1
0.7
SIZE (kb)
3.7
1.1
0.7
oBS1
1.1
0.7

FIGURE 1.7

Sizes of MspI restriction fragments detected by the group 1 fragment probe in genomic clones used as markers in the genomic blot shown in Figure 1.8. The genomic clone digested to produce each fragment and the region which hybridizes to the G1F probe (in some cases part of two fragments) are indicated.

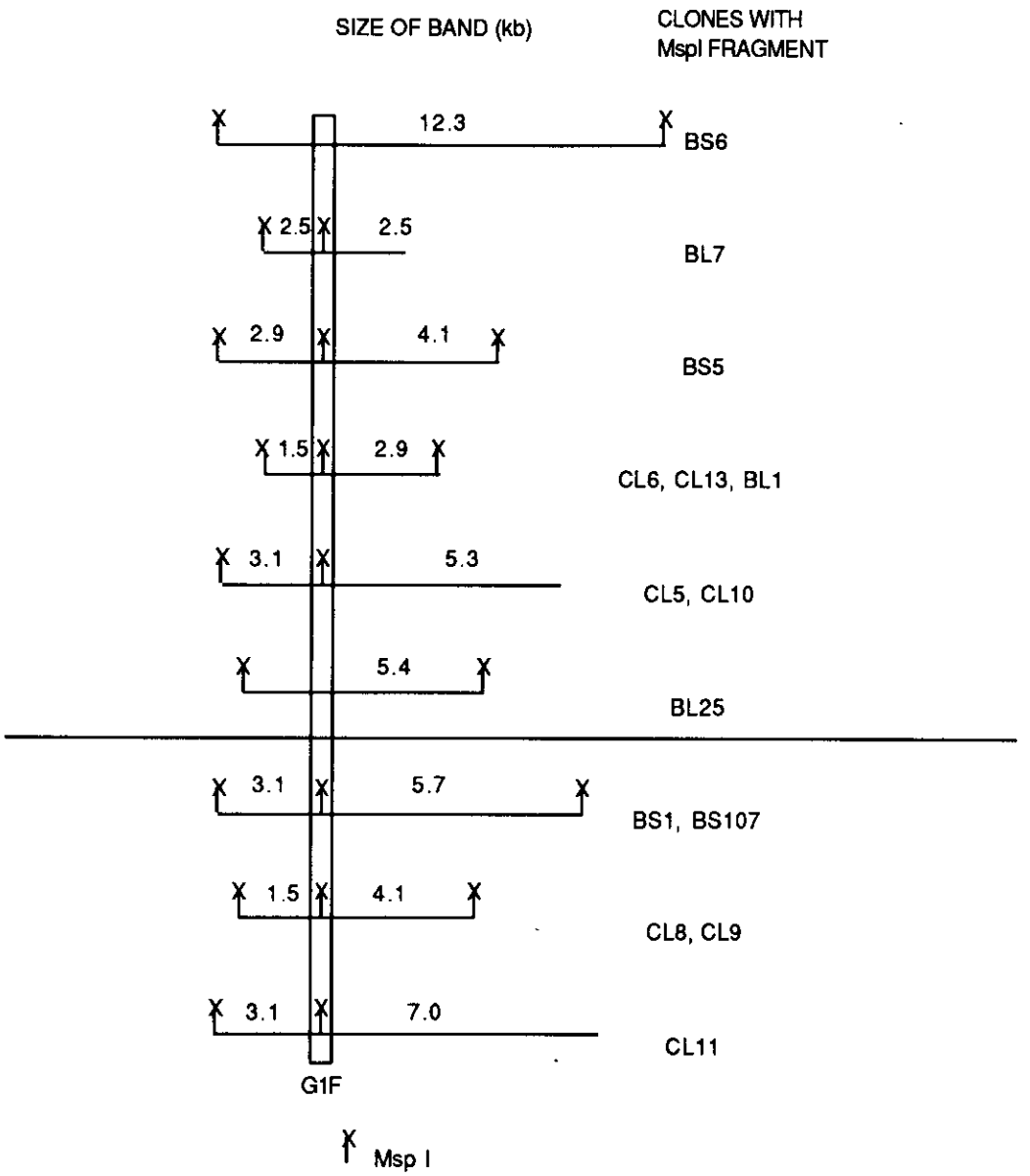
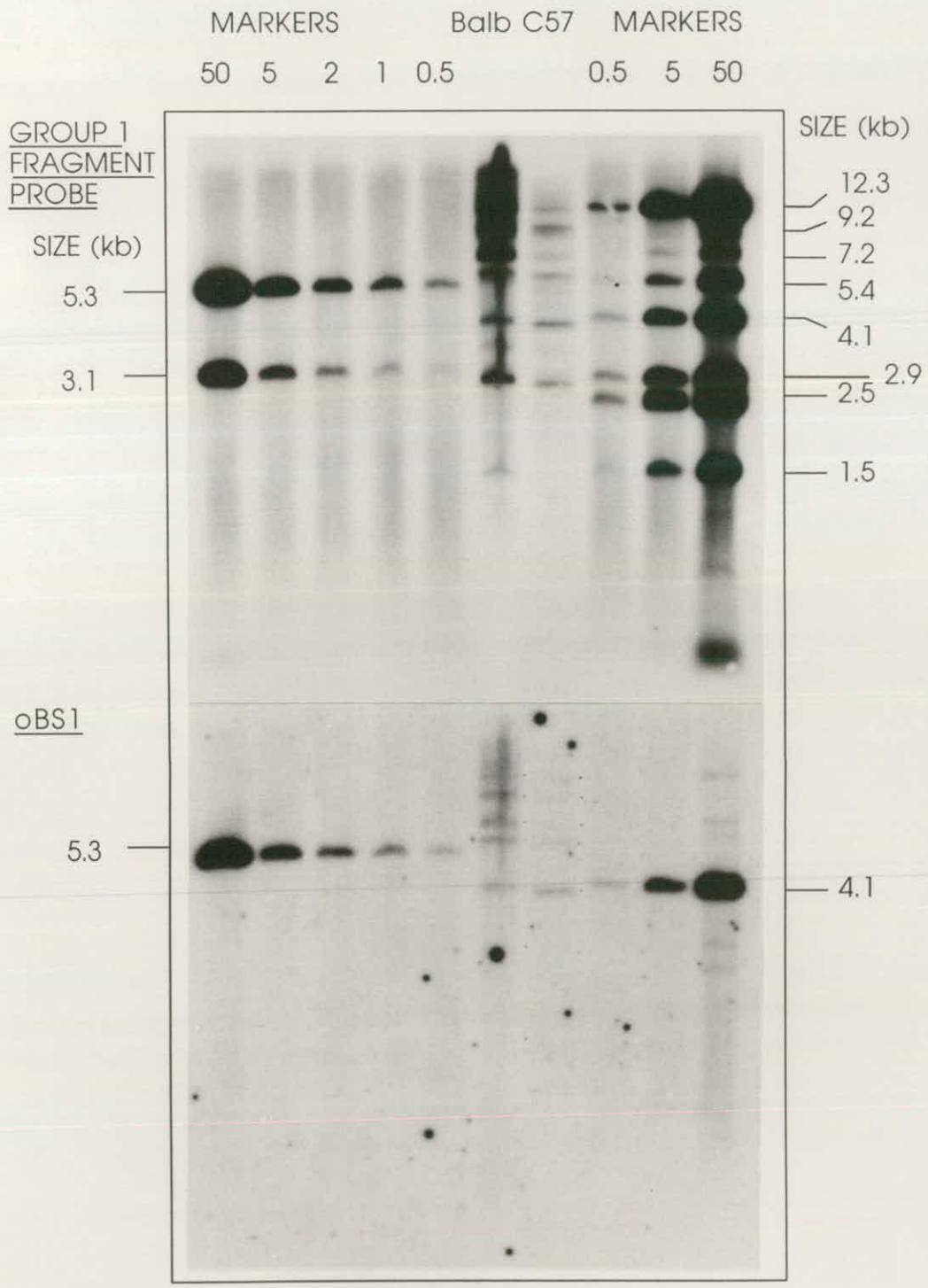

SIZE OF BAND (kb)
CLONES WITH
MspI FRAGMENT
12.3
BS6
2.5
2.5
BL7
2.9
4.1
BS5
1.5
2.9
CL6, CL13, BL1
3.1
5.3
CL5, CL10
5.4
BL25
3.1
5.7
BS1, BS107
1.5
4.1
CL8, CL9
3.1
7.0
CL11
G1F
Msp I

FIGURE 1.8

A Southern blot of Msp I digested genomic DNA hybridized with the oligonucleotide oBS1. Tracks contained 10µg of Msp I digested Balb/c or C57BL/6 genomic DNA or marker DNA. The fragments present in marker tracks are shown in the upper part of Figure 1.7. On the left hand side marker tracks contained the equivalent of 50, 5, 2, 1 and 0.5 copies and on the right hand side the equivalent of 0.5, 1, 2, 5 and 50 copies. The gel was 0.6% agarose.

The filter was hybridized twice, firstly to the oligonucleotide oBS1 (bottom panel) and then to the group 1 fragment probe (top panel). The filters were exposed for 1 hour at room temperature (top panel) or 3 weeks at -70°C with screens (bottom panel).


MARKERS
Balb C57
MARKERS
50 5 2 1 0.5
0.5 5 50
GROUP 1 FRAGMENT PROBE
SIZE (kb)
5.3
3.1
SIZE (kb)
12.3
9.2
7.2
5.4
4.1
2.9
2.5
1.5
oBS1
5.3
4.1

DNA appeared to be under-loaded. Bands of 2.9, 4.1, 5.6, 7.2, 10 and 12.3kb are detected with the G1F probe in genomic DNA from both strains. A 1.5kb band is present in both Balb/c and C57BL/6 genomic DNA but is only detected in the C57BL/6 track when the autoradiograph is exposed for longer than that shown. Additional faint bands are seen in DNA from both strains.

In the marker tracks, only two fragments hybridize to the oligonucleotide oBS1, a 5.3kb fragment from CL5 and a 4.1kb fragment from BS5 (Figure 1.8). In genomic DNA from both Balb/c and C57BL/6, five fragments (4.1, 5.4, 7.2, 10 and 12.3kb) hybridize to the oligonucleotide which derive from at least five different group 1 genes. The fragments are the same size in both strains although the amount of each fragment detected is different. The 4.1 and 5.4kb fragments were detected at the same level in both strains. The 7.2, 10 and 12kb fragments were detected at a higher level in Balb/c DNA. Therefore there is a difference between the number of genes contributing each fragment in the two strains. This is consistent with previous work using a different group 1 probe (Al-Shawi, 1985).

DISCUSSION

Three oligonucleotides oBL1, oBL1RI and oBS1 were designed to distinguish *Mup-1.1B*, *Mup-1.1C*^{b} and *Mup-1.1C*^{a} mRNA in order to study the expression of four single copy group 1 genes in Balb/c and C57BL/6 mice. The group 1 MUP genes are highly homologous and, as mentioned, the sequence of only five Balb/c and one C57BL/6 group 1 coding regions are known. Hence the oligonucleotides were hybridized to genomic DNA in order to determine whether they would hybridize to any unknown group 1 genes and therefore to more than one MUP mRNA.

oBL1 and oBL1RI, which were designed to detect *Mup-1.1C* mRNA, did not hybridize to any unknown group 1 genes. oBL1 could only hybridize to *Mup-1.1C*^{a} and not *Mup-1.1C*^{b}. oBL1RI could hybridize to *Mup-1.1C*^{a} and *Mup-1.1C*^{b} but also cross-hybridized to non-MUP sequences in genomic DNA. The origin of this hybridization is not known. oBL1 hybridizes to just one group 1 gene, *Mup-1.1C*^{a} and it can potentially be used to distinguish *Mup-1.1C*^{a} mRNA from all other group 1 mRNA in Balb/c liver. The oligonucleotide oBL1RI hybridized to two group 1 genes, *Mup-1.1C*^{a} and *Mup-1.1C*^{b}, one from each of two inbred strains of mice. oBL1RI can be used to compare the expression of *Mup-1.1B*^{a} and *Mup-1.1B*^{b} in Balb/c and C57BL/6.

oBL1 can distinguish mRNA from *Mup-1.1C*^{a} and *Mup-1.1C*^{b}. This is potentially useful to enable the expression of the two genes to be studied in recombinant inbred (RI) strains or in F_1 hybrids between Balb/c and C57BL/6. Previous experiments using recombinant inbred (RI) strains or F_1 hybrids showed that secretion of MUP proteins in urine or the level of MUP mRNA is modified by a number of *trans*-acting loci (Szoka and Paigen, 1978; Berger and Szoka, 1981; Ma, 1987). The oligonucleotides oBL1 and oBL1RI could be used to determine whether these *trans*-acting loci affect the level of specific group 1 mRNAs.

The MUP gene family arose by duplication after which mutations occurred which then spread throughout the family by unequal crossing over or gene conversion (Al-Shawi *et al.*, 1989). *Mup-1.1C*^{a} and *Mup-1.1C*^{b} are almost identical but both are different from many other MUP genes in the same strains (Al-Shawi *et al.*, 1989; Held *et al.*, 1987). The close relationship of *Mup-1.1C*^{a} and *Mup-1.1C*^{b} suggests that both are descended from the same gene. The single mutation in the coding region which distinguishes *Mup-1.1C*^{a} is not found in any other MUP gene. Unless it is conserved by selection, one would hypothesize that this

mutation would eventually either spread to the other group 1 genes or be lost.

oBS1, which was designed to detect *Mup-1.1B*, hybridized not only to *Mup-1.1B* but also to several other group 1 genes in both strains of mice. oBS1 hybridized to four group 1 genomic clones of which BS1, CL5 and CL10, are clones of *Mup-1.1B* whereas BS5 is not. Mapping experiments were carried out on the genes which hybridized to oBS1. Genomic DNA from the two strains was digested with three enzymes; EcoRI, PstI and MspI. In two of these digests there was a deference between the fragments detected in Balb/c and C57BL/6 DNA. The results showed that a 0.7kb fragment was detected in Pst I digested C57BL/6 DNA but not Balb/c DNA. Using a different probe, Al-Shawi (1985) detected a 0.7kb Pst I fragment in both strains. This difference could be due to the ability of the previous probe (which was longer than 0.7kb) to hybridize with another fragment in Balb/c genomic DNA. The Pst I site in intron 2 responsible for generating the 0.7kb fragment may have been involved in a gene conversion event (Al-Shawi, 1985). *Mup-1.1B*^{b} and *Mup1.1B*^{a} are closely related but the Pst I site in intron 2 is only present in *Mup-1.1B*^{b}. The site could have been introduced into several C57BL/6 genes including *Mup-1.1B*^{b} or removed from several Balb/c genes including *Mup 1.1B*^{a} by a gene conversion event.

The strain difference observed in genomic DNA digested with Msp I was quantitative rather than qualitative. Quantitative differences in the hybridization to fragments in Msp I digested DNA from both strains was previously observed using another probe (Al-Shawi, 1985). The location of Msp I sites may differ in closely related genes more than the other restriction sites because the recognition sequence for MspI contains a CpG methylation sequence. This may lead to frequent mutation of ^{m}C to T and thus loss of Msp I sites. Five different fragments in Msp I

digested DNA hybridized with the oBS1 probe which indicates that at least five different genes are detected with this oligonucleotide.

McIntosh and Bishop (1989) hybridized oBS1 to Balb/c liver RNA in order to determine whether the expression of *Mup-1.1B*[a] was different in males and females. mRNA which hybridized with oBS1 was present in male liver RNA at one third the level of *Mup-1.1C*[a] RNA (which was detected with the oligonucleotide oBL1). In female Balb/c liver the level of hybridization with oBS1 was extremely low, whereas the level of *Mup-1.1C*[a] mRNA in females was only about eleven fold lower than that in males. They concluded that mRNA detected with oBS1 was much more sexually dimorphic (250 times) than *Mup-1.1C*[a] RNA (11 times). Therefore oBS1 hybridizes to a very sexually dimorphic MUP mRNA in Balb/c mice. The results shown here indicate that up to five different group 1 genes may contribute to this sexually dimorphic mRNA.

Al-Shawi *et al.*, (1989) suggested that closely related MUP genes formed sub-families which had the same pattern of expression. Genes which hybridize to oBS1 may be members of one such sub-family. Alternatively some of the five genes may be comparatively silent. Shi *et al.*,(1989) suggest that many of the twelve to fifteen group 1 genes are silent. In the Balb/cJ substrain half of the group 1 MUP genes are deleted and none of the remaining six to eight are expressed (Duncan *et al.*, 1988; Shi *et al.*, 1989). There are two possible causes of this loss of group 1 expression. The functional group 1 genes could have been deleted leaving only non functional ones, alternatively the loss of expression may be due to the loss of an enhancer which affects the expression of all MUP genes. *Mup-1.1C*[a] is known to be capable of expression however the EcoRI fragment which is thought to be derived from *Mup-1.1C*[a] is not detected with a group 1 MUP probe in these mice which makes it very likely that this gene has been deleted.

As mentioned in the introduction, there are several methods available to distinguish between closely related sequences and it may be possible to use a different method to find out if other MUP mRNAs are present in the liver. Ribonuclease protection has the advantage over hybridization to oligonucleotides in that the probe covers a large section of mRNA. It is therefore possible to identify mismatches between mRNAs and the probe over the whole mRNA sequence. Other group 1 genes expressed in the liver might be identified in this way.

CHAPTER 2

IDENTIFYING THE PROTEIN PRODUCTS OF MUP GENES

If the protein product of each group 1 MUP gene can be identified then the different proteins synthesized in males and females or in mice treated with GH and thyroxine would be indicative of the genes being expressed. In this way genes which respond differently could be identified. There is no reason to suppose that genes with the same protein product are regulated in the same way, however, if a protein is not present in urine, it is likely that none of the genes which code for that protein are expressed. If a protein is present in urine it is not possible to determine the level at which each gene which codes for that protein is expressed and the products of each gene must be distinguished in some other way.

Many group 1 MUP genes have different coding sequences and, as mentioned in chapter 1, six different group 1 coding sequences are known, of which, five are from Balb/c and one is from C57BL/6. There are one or two nucleotide differences between the six coding sequences and in some cases this causes a difference of a single amino-acid in the protein. MUPs which differ by a single amino-acid can be separated by narrow range IEF providing that the amino-acid change affects the isoelectric point. By a combination of IEF and SDS PAGE, seven different MUPs have been identified in C57BL/6 urine (Kuhn *et al.*, 1984). MUPs present in urine are synthesized in the liver (Finlayson *et al.*, 1965) and hybrid selection was used to separate group 1 and group 3 liver mRNA and thus identify which proteins in urine are products of the two groups of genes (Kuhn *et al.*,

1984). The five proteins synthesized *in vitro* from group 1 mRNA were identical to five proteins in urine named MUPs 1 to 5. The four or more proteins synthesized *in vitro* from group 3 mRNA (which codes for a protein with an N-linked glycosylation site) were glycosylated and could easily be distinguished from the group 1 proteins (Ma, 1987; Kuhn *et al.*, 1984). These were referred to as MUP 6.

TABLE 2.1 Proteins coded by MUP genes

GENOMIC CLONE	MOUSE STRAIN	PROTEIN
BL6-25	C57BL/6	MUP2
BL6-51	C57BL/6	MUP2
BL6-11	C57BL/6	MUP3
BL6-3	C57BL/6	MUP3
BL6-42	C57BL/6	MUP4
BJ-31	BALB/C	MUP2a

Different sets of MUPs are synthesized in Balb/c and C57BL/6 (Clissold and Bishop, 1982). The glycosylated MUPs appear to be identical in the two strains (Sampsell and Held, 1985; Kuhn *et al.*, 1984; Ma, 1987) and the differences are due to group 1 genes. In Balb/c there are three group 1 MUPs and these have similar isoelectric points to the C57BL/6 MUPs 2, 4 and 5 (Sampsell and Held, 1985; Clissold and Bishop, 1982).

Held *et al.* (1987) identified the products of MUP genomic clones from C57BL/6 and Balb/c by transfecting MUP clones into mouse L-cells in tissue culture and isolating the secreted protein from the cell medium. The products which they identified are shown in Table 2.1. In the work described here, the protein products of three Balb/c group 1 genes and one group 3 gene were identified. Proteins made by males and females were compared so that inferences could be drawn about the expression of these four genes.

RESULTS

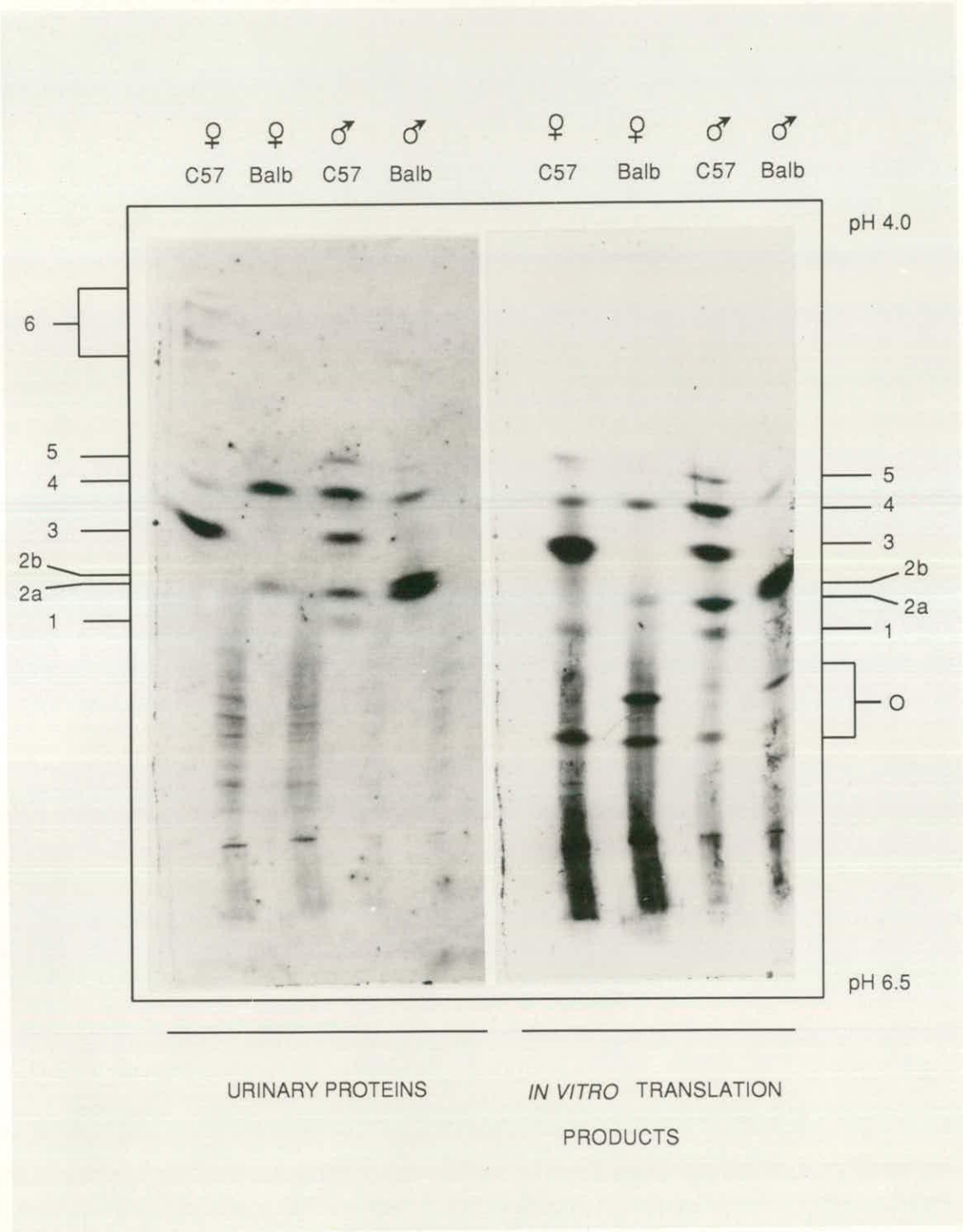
The proteins made *in vitro* from total mouse liver mRNA and proteins isolated from urine of a mouse of the same sex and strain were compared by loading them in adjacent tracks on one dimensional IEF gels (Figure 2.1). In agreement with previous work (Knopf *et al.*, 1983), MUPs 1 to 5 synthesized *in vitro* comigrated with MUPs 1 to 5 from urine, whereas MUP 6 from urine which has been shown to be glycosylated (Ma, 1987; Kuhn *et al.*, 1984) was not found amongst MUPs synthesized *in vitro*. The alternatively glycosylated MUPs synthesized *in vitro* can be identified on the basis of their molecular weight (Figures 2.5 and 2.7). These MUPs are discussed later. Several proteins (O, Figure 2.1) were synthesized *in vitro* which did not comigrate with MUPs from urine. These may be other liver proteins as they were not observed when hybrid selected MUP mRNA rather than total liver mRNA was translated (Knopf *et al.*, 1983).

cDNA clones of three group 1 genes and one group 3 gene from Balb/c were used to make MUP proteins. The origins of the four cDNA clones and the genomic clones with the same coding sequence are shown in Table 2.2. The

FIGURE 2.1

Isoelectric focusing gel showing the comigration of urinary proteins (left) with proteins synthesized *in vitro* from liver RNA (right). Tracks contained 10µg of urinary protein or 210,000cpm (female), 41,000cpm (male) synthesized proteins labeled with ^{35}S methionine. The gel was stained with coomassie blue, dried, then autoradiographed for 44 days at -70°C with screens.

In vitro translation products made from liver RNA from Balb/c and C57BL/6 males and females contain different amounts of MUP4. In the male products the level of MUP 4 is lower in Balb/c than C57BL/6 whereas in the female products it is lower in C57BL/6. Different genes code for MUP4 in each strain and they appear to be regulated differently.


♀ C57
♀ Balb
♂ C57
♂ Balb
♀ C57
♀ Balb
♂ C57
♂ Balb
pH 4.0
6
5
4
3
2b
2a
1
5
4
3
2b
2a
1
O
pH 6.5
URINARY PROTEINS
IN VITRO TRANSLATION PRODUCTS

cDNA clones were subcloned into vectors containing the T7 RNA polymerase promoter. Maps of the constructs and the size of the run-off transcripts from the T7 promoter after linearization using BamHI are shown in Figure 2.2.

TABLE 2.2 Origins of MUP cDNA clones

NAME OF cDNA CLONE	GROUP	ORIGIN OF cDNA CLONE	GENOMIC CLONE WITH SAME CODING SEQUENCE
cLiv1	1	Shahan *et al.*, 1987a	BS1
cLiv6	1	Shahan *et al.*, 1987a	BL1
MUP 11 (cBS6)	1	Chave-Cox, 1986	BS6
MUP15	3	Chave-Cox, 1986	-

IEF was used to identify the proteins from *in vitro* translated liver mRNA which comigrated with the proteins from *in vitro* translated run-off transcripts (Figures 2.3 and 2.4). The correspondence between group 1 genes and proteins are shown in Table 2.3. The synthesis of these proteins is regulated in different ways. MUPs 2a and 4 are produced in both male and female Balb/c liver whereas MUP2b is produced only in males. This suggests that the genes BL1 and BS6 might be expressed differently to BS1 in Balb/c mice.

FIGURE 2.2

MUP cDNA clones in vectors containing the T7 RNA polymerase promoter. The size of run-off transcripts are indicated. cLiv1 and cLiv6 were cloned into pTZ18R (Mead and Szczesna-Skorupa, 1986) as EcoRI fragments. An EcoRI/BamHI fragment from MUP11 (Chave-Cox, 1986) was substituted for an EcoRI/BamHI fragment from cLiv1 to create cBS6 which has the same coding sequence as the genomic clone BS6. MUP 15 was cloned into pT7-1 (Tabor and Richardson, 1985) as an EcoRI/BamHI fragment by Ma (1987).

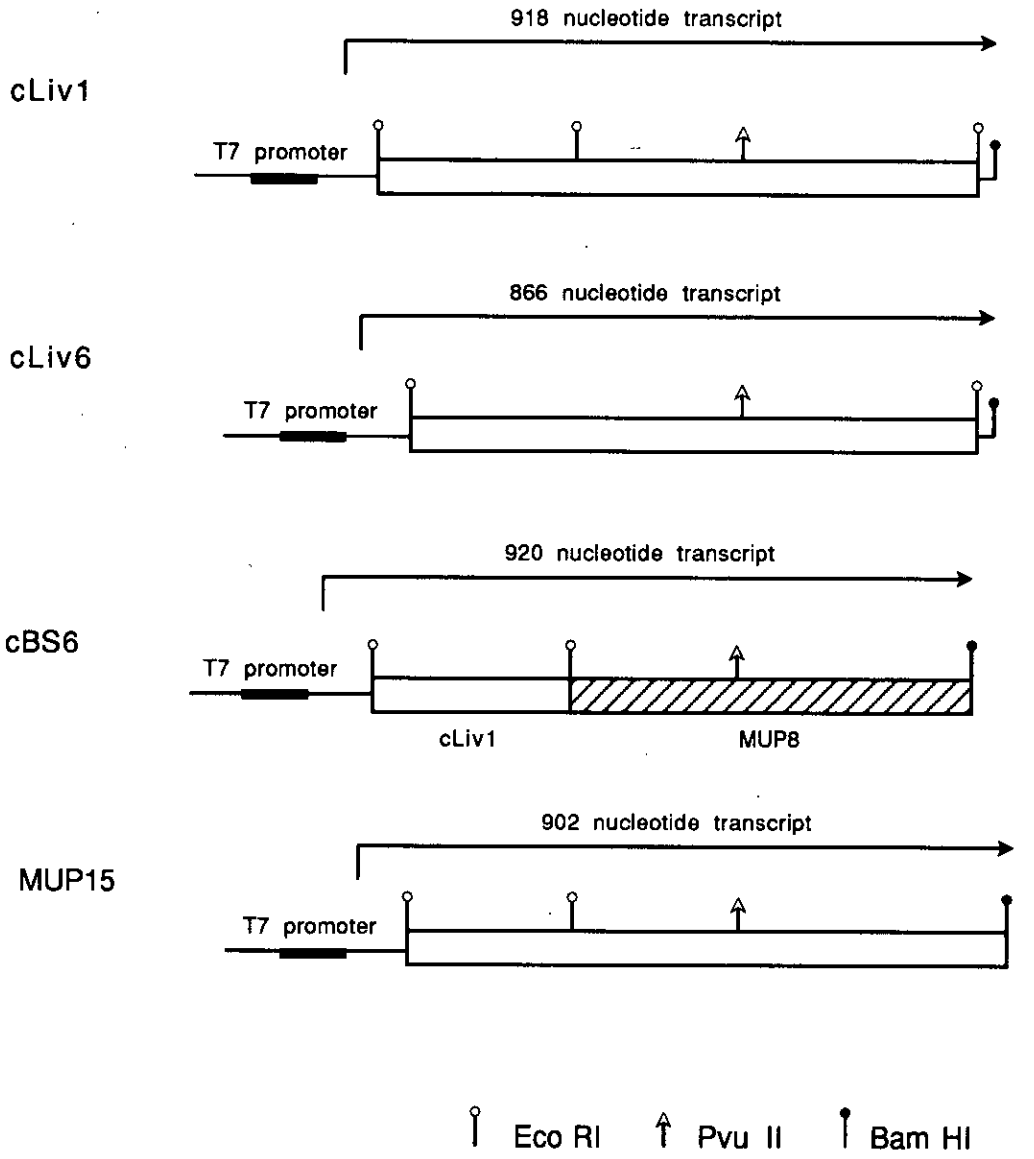

cLiv1
918 nucleotide transcript
T7 promoter
cLiv6
866 nucleotide transcript
T7 promoter
cBS6
920 nucleotide transcript
T7 promoter
cLiv1
MUP8
MUP15
902 nucleotide transcript
T7 promoter
Eco RI
Pvu II
Bam HI

FIGURE 2.3

An isoelectric focusing gel showing the comigration of individual MUP proteins made *in vitro* with proteins present in urine. Male C57BL/6 (1) and Balb/c (3) urinary proteins are compared with proteins synthesized *in vitro* from male C57BL/6 liver RNA (2 and 9), male Balb/c liver RNA (4 and 7), cLiv1 RNA (5), cLiv6 RNA (6) or mup15 RNA (8). 42,000cpm were loaded in tracks 2, 4, 7 and 9, 10,000cpm in tracks 5 and 6, and 20,000cpm in track 8. The gel was stained with coomassie blue, dried, then autoradiographed for 10days at -70°C with screens.

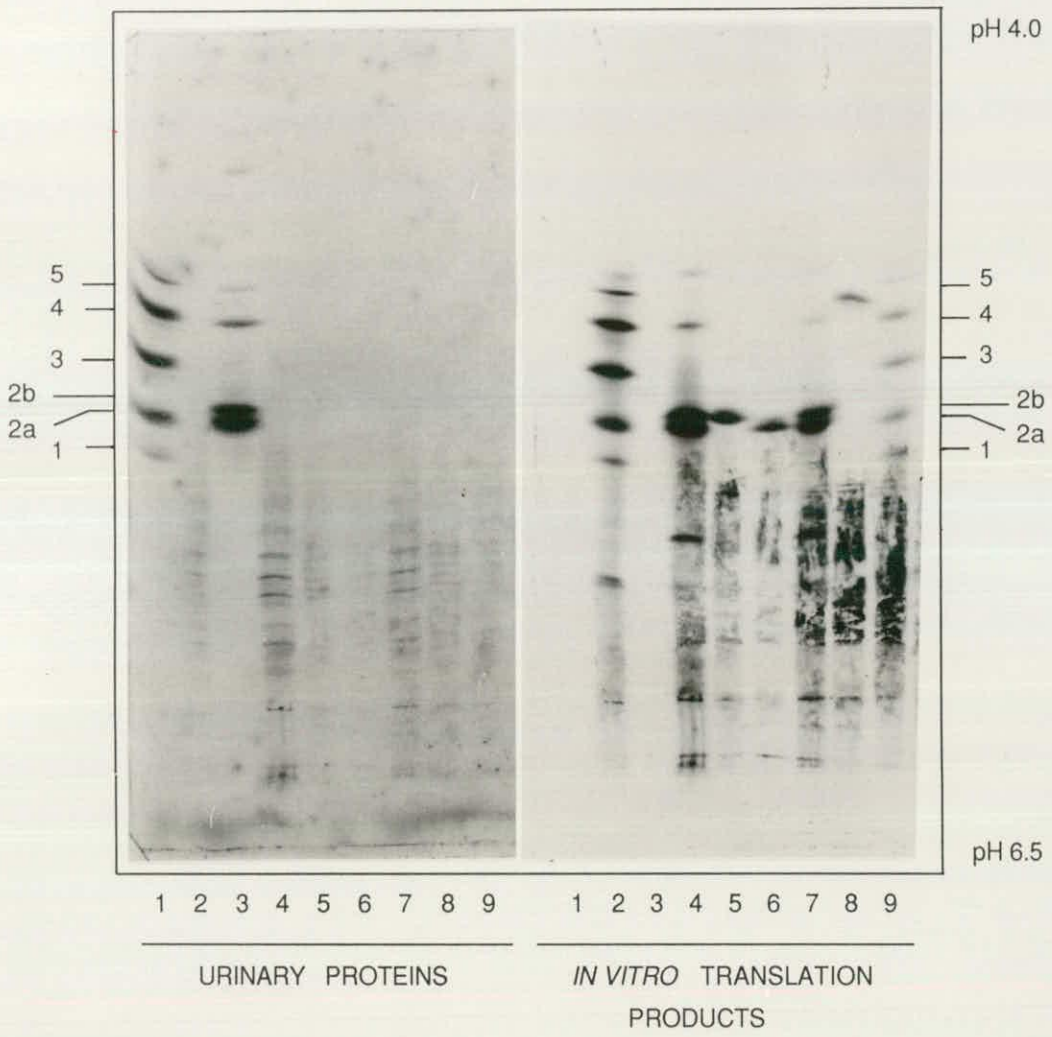

pH 4.0
5
4
3
2b
2a
1
pH 6.5
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
URINARY PROTEINS
IN VITRO TRANSLATION PRODUCTS

FIGURE 2.4

An isoelectric focusing gel showing the comigration of individual MUP proteins made *in vitro* with proteins present in urine. Male and female C57BL/6 and Balb/c urinary proteins (left) are compared with proteins synthesized *in vitro* from male C57BL/6 liver RNA, male Balb/c liver RNA and cBS6 RNA in presence (+) or absence (-) of membranes. 40,000cpm of liver translation products and 10,000cpm of BS6 protein were loaded. The gel was stained with coomassie blue, dried, then autoradiographed for 7 days at -70°C with screens.

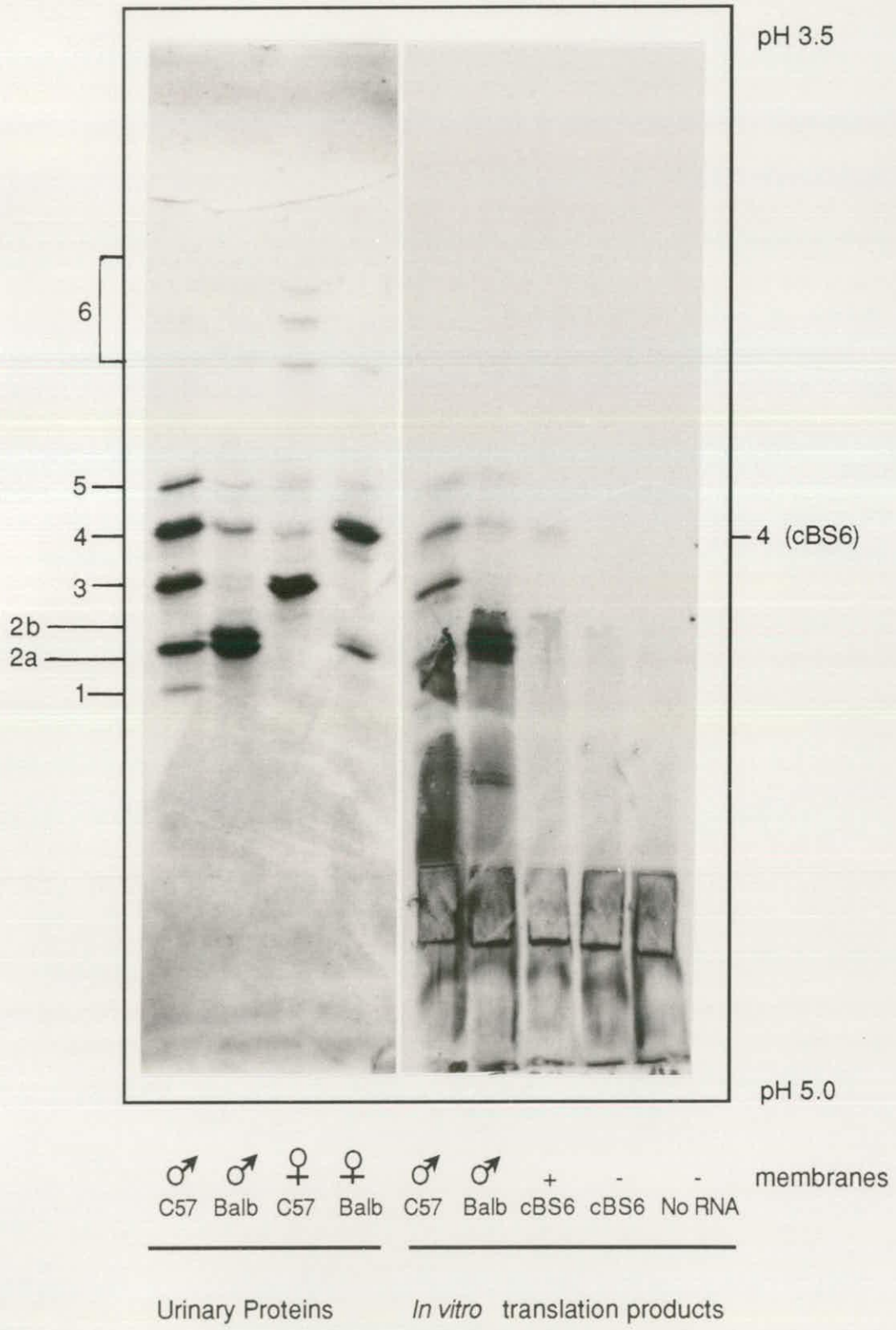

pH 3.5
6
5
4
3
2b
2a
1
4 (cBS6)
pH 5.0
♂ C57
♂ Balb
♀ C57
♀ Balb
♂ C57
♂ Balb
+ cBS6
- cBS6
- No RNA
membranes
Urinary Proteins
In vitro translation products

TABLE 2.3 Correspondence between cDNA clones and proteins

cDNA CLONE	GENOMIC CLONE WITH SAME CODING SEQUENCE	PROTEIN
cLiv1	BS1	MUP2b
cLiv6	BL1	MUP2a
cBS6	BS6	MUP4

On IEF gels, the protein from *in vitro* translated run-off transcripts from the group 3 clone MUP15 did not comigrate with any *in vitro* translated proteins from liver RNA (Figure 2.3 track 8). This suggests that the protein is synthesized from a minor liver mRNA and is not detected on this gel. *In vivo* the group 3 mRNAs are thought to code for MUP 6 which is not synthesized *in vitro* from liver mRNA, probably because of glycosylation differences *in vitro* and *in vivo*. There are two types of post-translational modifications which affect MUP proteins, cleavage of the signal peptide (Szoka *et al.*, 1980) and glycosylation. The signal peptides vary from 22 amino-acids in the MUP15 protein to 18 amino-acids in most group 1 MUPs. Only group 3 MUPs have a glycosylation signal.

The proteins synthesized in reticulocyte lysates are unprocessed primary translation products. When canine pancreatic microsomal membranes are added during translation the signal peptides are cleaved off and some glycosylation steps are performed. The addition of membranes during the translation of mRNA from group 1 genes leads to the processing of the MUP

precursor (3, Figures 2.5 and 2.6) to give a lower molecular weight protein which is the same size as the majority of MUPs found in urine. This is consistent with the removal of the signal peptide. On the other hand the addition of membranes during the translation of mRNA from MUP 15 leads to the production of a higher molecular weight protein (2) (Figure 2.5). This is consistent with glycosylation (Rothman and Lodish, 1977; Goldman and Blobel, 1981). Proteins (6) and (7) in Figure 2.5 are thought to be cleaved derivatives of (2).

In order to determine whether the higher molecular weight protein is present in *in vitro* translated liver mRNA, the proteins were subjected to two dimensional gel electrophoresis (Figure 2.7). The first dimensional narrow range isoelectric focusing from pH 4 to 6.5 excludes almost all other liver proteins. The second dimension was SDS PAGE where the glycosylated MUPs are separated from non-glycosylated MUPs by molecular weight (Figure 2.7a (6)). When MUPs made *in vitro* from MUP 15 RNA were run on two dimensional gels, four proteins were detected (Figure 2.7f). There was a single higher molecular weight protein which suggests that MUP 15 mRNA can be translated to give a single glycosylated product. There were three lower molecular weight proteins which may be partially processed. In *in vitro* translated liver mRNA from either Balb/c or C57BL/6 (Figure 2.7b and d), two higher molecular weight proteins were detected. The fact that MUP 15 mRNA gave only one product suggests that these two proteins are translated from two different group 3 mRNAs. Both male and female *in vitro* translated liver mRNA (data not shown) contained two higher molecular weight proteins indicating that the same group 3 proteins are made in the livers of male and female mice.

Two differences were observed between the proteins made *in vitro* and

FIGURE 2.5

15% SDS PAGE showing the molecular weight of MUPs translated *in vitro* from male C57BL/6 or Balb/c liver mRNAs or individual MUPs (cLiv1, cLiv6 and Mup15) in the presence (+) or absence (-) of membranes. The processed MUPs (4) were identified by their comigration with proteins from male Balb/c urine and by molecular weight markers run in tracks on either side of the gel (not shown).

Protein (1) is the product of endogenous RNA present in the reticulocyte lysate. Protein (5) comigrates with haemoglobin. Proteins 2 to 4, 6 and 7 are products of MUP mRNA.

The number of counts loaded were 42,000 cpm (Male C57BL/6 (-) and (+) and Balb/c (-)), 21,000 cpm (Balb/c (+)) and 10,000 cpm (individual MUPs). The gel was soaked in amplify (Amersham) after coomassie blue staining and autoradiographed for 7 days at -70°C with screens. A shorter exposure showed bands 3 and 4 in track 2, which may be due to incomplete processing of the male C57BL/6 proteins as suggested in figure 2.7.

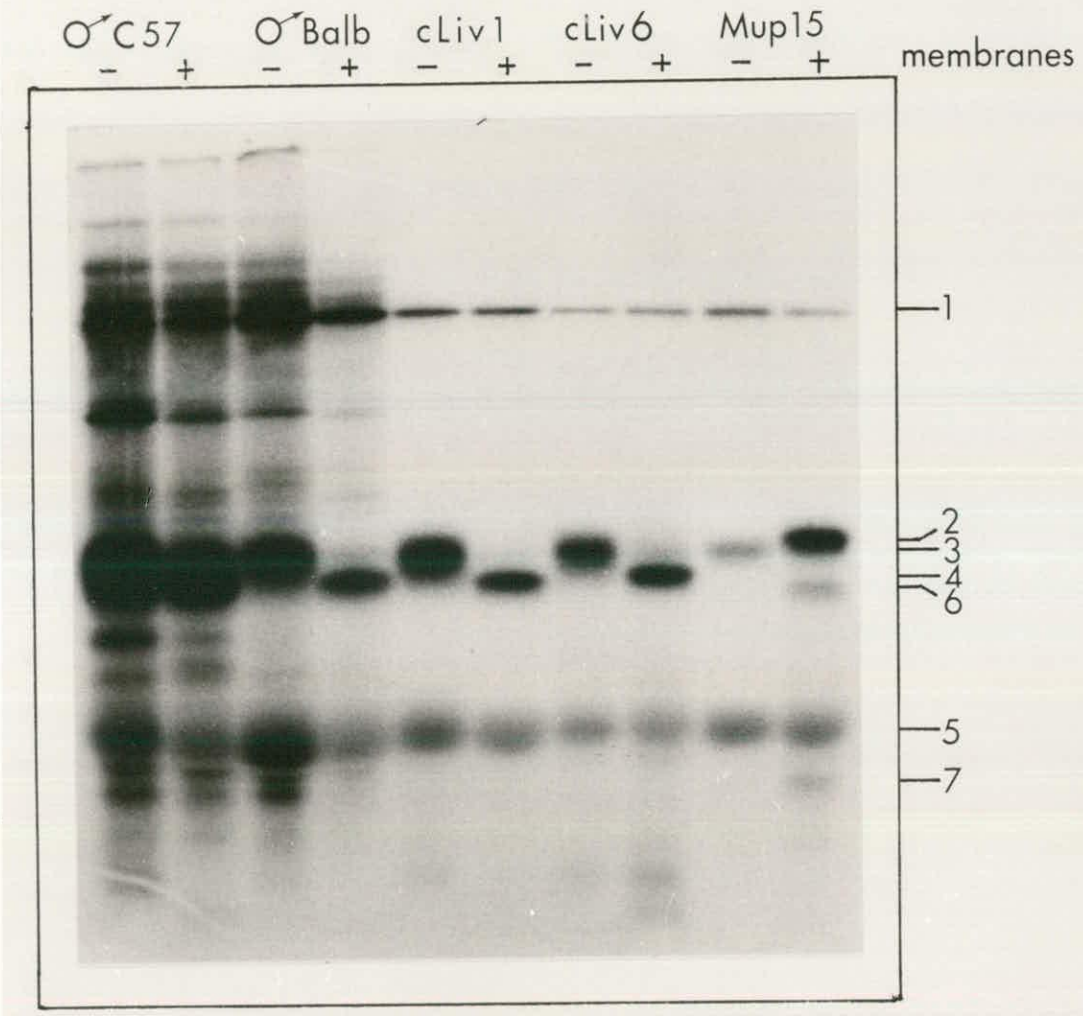

♂C57
♂Balb
cLiv1
cLiv6
Mup15
− + − + − + − + − +
membranes
1
2
3
4
6
5
7

FIGURE 2.6

A 15% SDS PAGE showing the molecular weight of MUPs translated *in vitro* from male C57BL/6 or Balb/c liver mRNA, cBS6 RNA or no RNA in the presence (+) or absence (-) of membranes. The processed MUPs (4) were identified by their comigration with proteins from male Balb/c urine and by molecular weight markers run in tracks on either side of the gel (not shown).

Proteins (3) and (4) are unprocessed (3) or processed (4) products of MUP RNA. Without the addition of RNA protein (1) is translated and is therefore the product of endogenous RNA present in the reticulocyte lysate. Protein (5) comigrated with haemoglobin.

The number of counts loaded were 40,000cpm (C57BL/6 and Balb/c), 10,000cpm (cBS6 and NO RNA). After staining with coomassie blue the gel was soaked in amplify (Amersham) and autoradiographed for 4 days at -70°C with screens.

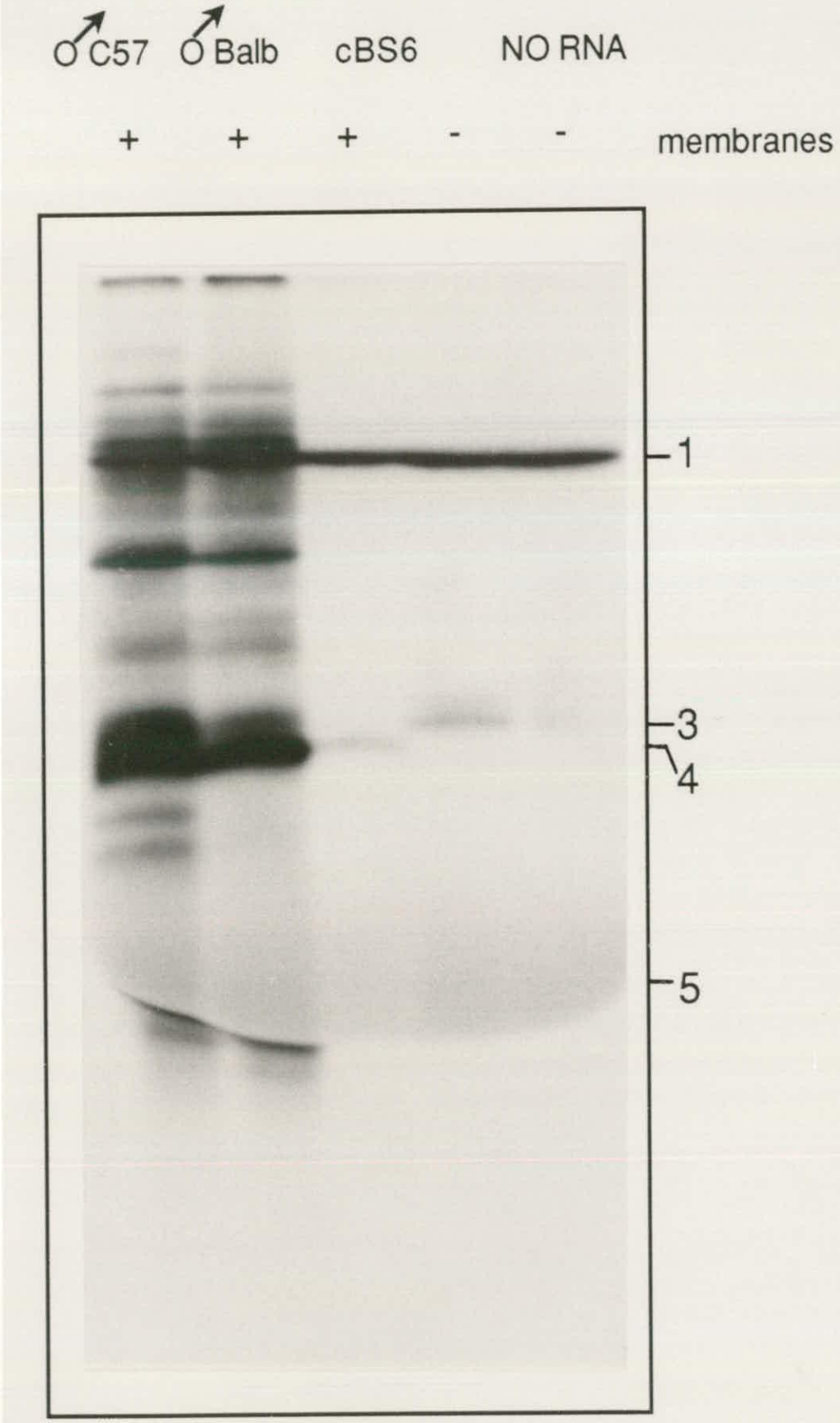
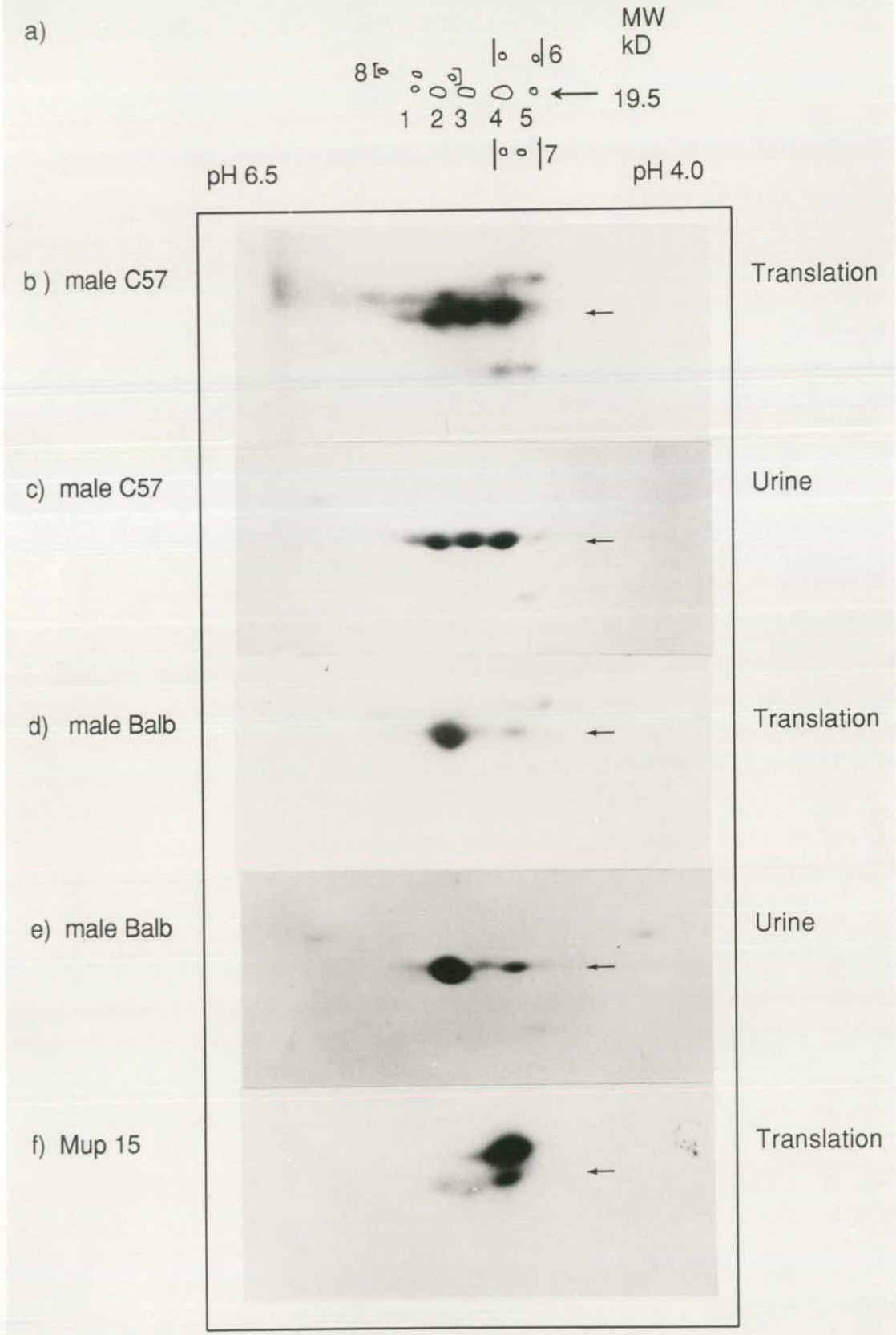

♂C57
♂Balb
cBS6
NO RNA
+
+
+
-
-
membranes
1
3
4
5

FIGURE 2.7

Two dimensional protein gels showing the molecular weight and isoelectric points of MUPs from Male C57BL/6 and Balb/c urine (c and e) or translated *in vitro* (in the presence of membranes) from male C57BL/6 or Balb/c liver mRNAs (b and d) or Mup15 RNA (f). The proteins were separated first by isoelectric focusing (horizontal) and then by molecular weight (vertical). The nomenclature of MUPs (panel (a)) is as Knopf *et al.*, 1983).

The gels were loaded with 40µg of protein from urine and either 84,000cpm (C57BL/6 and Balb2/c) or 20,000cpm (Mup15). The gels were stained with coomassie blue, soaked in amplify (Amersham), dried, autoradiographed at for 7 to 14 days at -70°C with screens (b, d and f).


a)
MW
kD
8
6
19.5
1 2 3 4 5
7
pH 6.5
pH 4.0
b) male C57
Translation
c) male C57
Urine
d) male Balb
Translation
e) male Balb
Urine
f) Mup 15
Translation

urinary proteins. Proteins (6) (Figure 2.7a) are higher molecular weight proteins which are probably glycosylated differently *in vitro* and *in vivo* (see above). Proteins (8) (Figure 2.7a) are present in low amounts in *in vitro* translation products and are thought to be unprocessed or partially processed versions of MUPs 1 to 5. These proteins were the same size as unprocessed MUPs and were more basic than processed MUPs consistent with the presence of the leader sequence.

DISCUSSION

The protein products of three group 1 Balb/c MUP genes and one group 3 MUP gene were identified. The proteins made from three Balb/c genomic clones, BS1, BL1 and BS6 are MUPs 2a, 2b and 4 respectively. The protein made by the group 3 gene was a glycosylated MUP. Table 2.4 shows a summary of the protein products of nine group 1 genomic clones from Balb/c and C57BL/6 including those identified by Held *et al.* (1987), Table 2.5 shows a summary of the group 1 subgroups. Genes in each subgroup code for protein products which have similar patterns of sexually dimorphic expression.

BL6-25 and BL6-51 were both isolated from a C57BL/6 library (Held *et al.*, 1987). It is thought that they are clones of the same gene because they code for the same protein (MUP2), have the same restriction map (Table 2.4) and are identical for 880bp of 5' proximal sequence (Held *et al.*, 1987). They are members of a group 1 sub-group called *Mup-1.1D* (Al-Shawi *et al.*, 1989; Table 2.5). MUP 2 is synthesized only in male liver.

BL1 and BJ-31 are clones of *Mup-1.1C*[a] from two different Balb/c substrains as mentioned in Chapter 1 (Table 1.1; Table 2.5) which code for Mup2a

TABLE 2.4

GENOMIC CLONE	cDNA CLONE	MOUSE STRAIN	PROTEIN	RESTRICTION MAPS OF GENOMIC CLONES
BL6-25	-	C57BL/6	MUP2	
BL6-51		C57BL/6	MUP2	SAME
BL1	cLiv6	BALB/C	MUP2a	
BJ-31		BALB/C	MUP2a	SAME
BS1	cLiv1	BALB/C	MUP2b	-
BL6-11		C57BL/6	MUP3	
BL6-3		C57BL/6	MUP3	DIFFERENT
BL6-42		C57BL/6	MUP4	
BS6		BALB/C	MUP4	DIFFERENT
-	Mup15	BALB/C, C57BL/6	MUP6	

(Table 2.4). Clones of the C57BL/6 allelic gene *Mup-1.1C*^{b} (Table 1.1) include BL6-11 the product of which is MUP3 (Table 2.4; Held *et al.*, 1987). Thus *Mup-1.1C*^{b} and *Mup-1.1C*^{a} code for different proteins both of which are present in the males and females of their respective strains (Table 2.5).

The C57BL/6 genomic clone BL6-3 has an identical map to the Balb/c genomic

clone BS6 and both clones are part of a group 1 subgroup called *Mup-1.1A* (Al-Shawi et al., 1989; Table 2.5). 570bp of the 5' proximal region of the two clones have been sequenced (Clark *et al.*, 1985; Held *et al.*, 1987). The two sequences differ by 7bp, 2bp of which are not in the hypervariable region whereas they differ from *Mup-1.1C*^{b} by 33bp, 9bp of which are not in the hypervariable region. BL6-3 codes for MUP3 which is present in male and female C57BL/6 urine (Held *et al.*, 1987). BS6 codes for MUP4 which is present in male and female Balb/c urine. Thus members of the *Mup-1.1A* subgroup appear to have the same pattern of sexually dimorphic expression.

The C57BL/6 genomic clone BL6-42 codes for MUP4 (Table 2.4) which is barely detectable in female C57BL/6 urine (Held *et al.*, 1987; Table 2.5). The Balb/c genomic clone BS1 codes for MUP2b (Table 2.4) which is barely detectable in female Balb/c urine. Both clones belong to a group 1 subgroup called *Mup-1.1B* (Table 2.5) and the members of this subgroup appear to have a highly sexually dimorphic pattern of expression.

There are two cases where a protein product corresponds to two group 1 genomic clones which are not identical and belong to two different subgroups. In one case a clone from each strain corresponds to MUP4 (Table 2.4). In the other case two C57BL/6 clones which are members two different subgroups (*Mup-1.1A*^{b} and *Mup-1.1C*^{b}; Table 2.4; Table 2.5), correspond to proteins that migrate as MUP3. The expression of MUP3 in C57BL/6 males and females (Table 2.5) could be due to the expression of either or both genes and in order to distinguish their expression a method other than identifying their protein products is required. This would allow the similar patterns of expression in males and females of members of the *Mup-1.1A*^{b} and *Mup-1.1C*^{b} subgroups to be verified.

In summary, four subgroups of group 1 MUP genes have been identified called *Mup-1.1A*, *B*, *C*, and *D*. In Balb/c and C57BL/6 the proteins corresponding to genes in the same subgroup had similar patterns of sexually dimorphic expression.

Group 3 MUP genes are expressed in the liver of both C57BL/6 and Balb/c mice and code for glycosylated proteins which are more acidic and higher in molecular weight than group 1 MUPs. *In vitro* transcribed group 3 RNA was translated *in vitro*. The group 3 protein was analysed by IEF and SDS PAGE and it was found to have a higher molecular weight than group 1 MUPs. It corresponded to one of two higher molecular weight products translated *in vitro* from both Balb/c and C57BL/6 liver RNA. This suggests that two different glycosylated proteins are translated *in vitro* from liver RNA. Only one type of group 3 cDNA clone has been isolated from C57BL/6 (Kuhn *et al.*, 1984) and Balb/c (Shahan *et al.*, 1987; Chave-Cox, 1986) however, there are at least two different group 3 genes present in Balb/c (Shi *et al.* 1989; Ma, 1987) and closely related sequences have been detected in C57BL/6 genomic DNA (Kuhn *et al.*, 1984). The results shown here suggest that at least two group 3 genes are expressed in the liver.

Table 2.5 Summary of group 1 subgroups and their protein products

GROUP 1 SUB-GROUP	STRA-IN	GENE	GENOMIC CLONES	PRO-TEIN	EXPRES-SION OF PROTEIN
Mup-1.1A	Balb	*Mup-1.1A*[a]	BS6	MUP4	M,F
	C57	*Mup-1.1A*[b]	BL6-3	MUP3	M,F
Mup-1.1B	Balb	*Mup-1.1B*[a]	BS1	MUP2b	M
	C57	*Mup-1.1B*[b]	BL6-42	MUP4	M
Mup-1.1C	Balb	*Mup-1.1C*[a]	BL1, BJ-31	MUP2a	M,F
	C57	*Mup-1.1C*[b]	CL6, CL13, BL6-11	MUP3	M,F
Mup-1.1D	C57	*Mup-1.1D*[b]	BL6-25/51	MUP2	M

CHAPTER 3

THE EXPRESSION OF MUP GENES IN THE LIVER OF BALB/C AND C57BL/6 MICE

Male Balb/c and C57BL/6 mice excrete different sets of MUPs (Szoka and Paigen, 1979; Berger and Szoka, 1981) the synthesis of which may be determined in three ways: 1) The two MUP alleles present in these strains contain distinguishable sets of coding sequences derived from distinct but closely related structural genes (Al-Shawi *et al.*, 1989; Chapter 1). 2) The structural genes may be linked to different *cis*-acting elements which affect their expression (Sampsell and Held, 1985). 3) Different *trans*-acting modifiers may be available in each strain (Duncan *et al.*, 1988; Szoka and Paigen, 1979; Berger and Szoka, 1981).

Different subgroups of the group 1 genes were shown to be regulated differently (Table 3.1; McIntosh and Bishop, 1989; Knopf *et al.*, 1983) and the sexual dimorphic expression of protein products of specific subgroups of group 1 MUP genes was shown to be similar (Table 2.5; Al-Shawi *et al.*, 1989). However, the expression of allelic subgroups in Balb/c and C57BL/6 mice has not been compared, neither has the expression of specific subgroups of group 1 genes been quantified in C57BL/6 mice. The levels of mRNAs in the liver made from two of the subgroups can be measured using oligonucleotide probes (Table 3.2; Chapter 1). The oligonucleotide oBL1RI hybridizes only to two genes; *Mup-1.1C*^{a} and *Mup-1.1C*^{b}. The proteins specified by *Mup-1.1C*^{a} and *Mup-1.1C*^{b} are expressed in both males and females which suggests that these allelic single copy genes might be regulated in the same manner in the two strains. However, as mentioned in chapter 2, the proteins specified by *Mup-1.1C*^{a}

and *Mup-1.1C*^{b} are also specified by other group 1 genes (BL6-3 and BL6-42) and a more accurate method of comparing the expression of *Mup-1.1C*^{a} and *Mup-1.1C*^{b} is to measure the mRNA levels in males and females of each strain using the oligonucleotide oBL1RI.

TABLE 3.1 Variation in MUP expression in the livers of Balb/c females

	mRNA in liver copies/cell				
	MALE	FEMALE			
PROBE		1	2	3	4
GROUP 1 cDNA	58,000	580	8,200	4,600	4,300
oBL1	39,000	230	9,100	2,900	1,700
oBS1	14,000	<15	150	48	25

The oligonucleotide oBS1 was previously shown to hybridize to a highly sexually dimorphic MUP mRNA in Balb/c liver (McIntosh and Bishop, 1989). Here the expression of C57BL/6 genes which hybridize with oBS1 is compared with the expression of the Balb/c genes. Recently it was shown that the MUP mRNA level in female Balb/c mice varies between individuals (Table 3.1, GROUP 1 cDNA) (McIntosh and Bishop, 1989). Similar variation was not observed between individual Balb/c males which means that sexually dimorphic expression is variable in Balb/c mice. In order to determine whether variable levels of MUP expression occur in individual Balb/c and C57BL/6 mice, MUP proteins were isolated from urine. Several urine samples can be taken from one mouse over a period of time so that it is possible to determine to what extent MUP expression in one mouse changes from day to day and hence to what

TABLE 3.2 Group 1 MUP genes

Mup gene designation	synthesis of protein Male(M), female(F)	oligonucleotide probes		
		oBL1RI	oBL1	oBS1
Mup-1.1B[a]	M	-	-	+
Mup-1.1B[b]	-	-	-	+
Mup-1.1C[a]	M F	+	+	-
Mup-1.1C[b]	M F	+	-	-

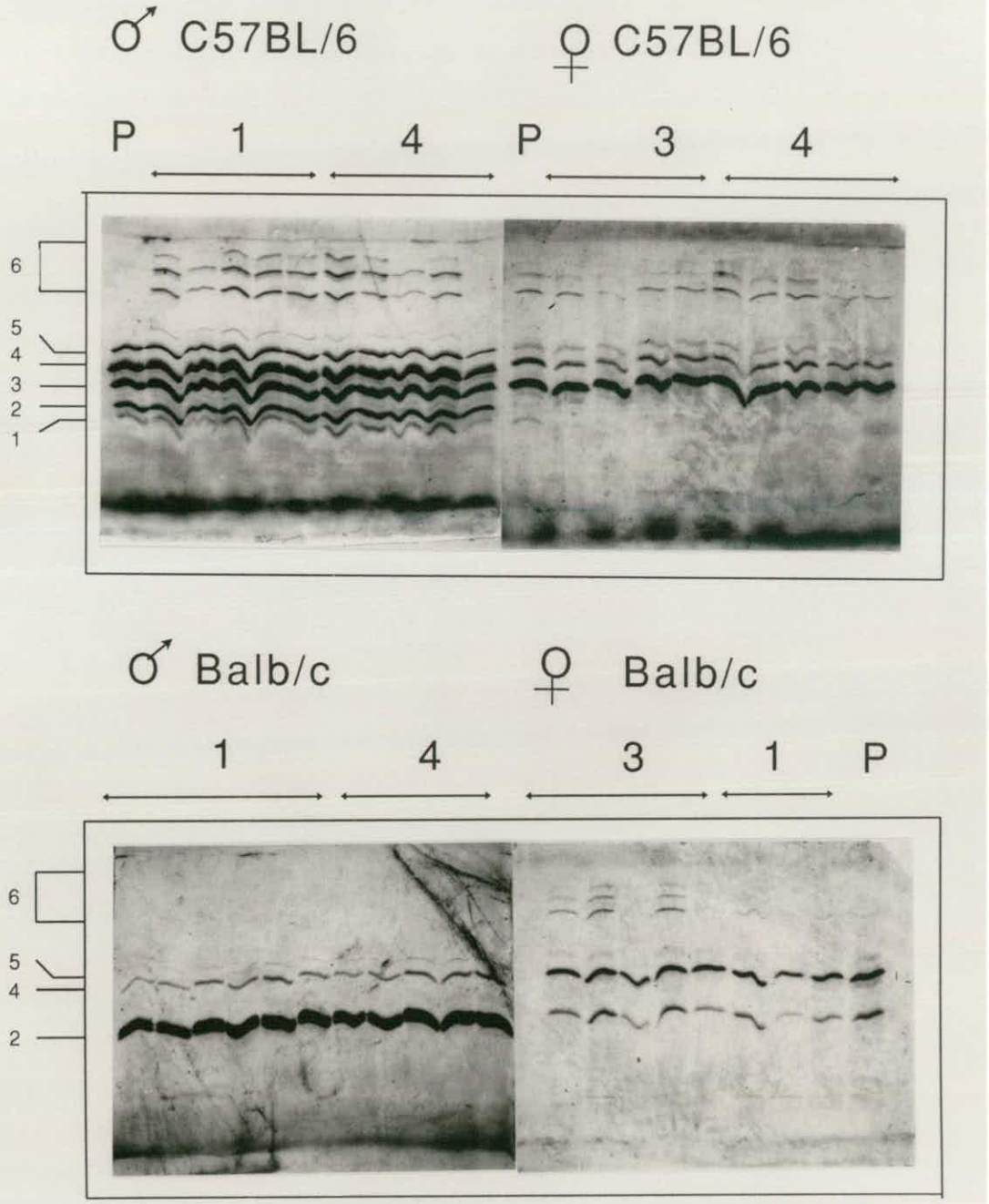
extent there is variation between mice. The group 1 genes and group 3 genes are coordinately regulated in the liver during development (Shahan *et al.*, 1987b) but they respond differently to hormonal stimuli (Kuhn *et al.*, 1984). The group 1 and group 3 proteins and mRNAs may vary differently between mice, hence the levels of group 1 and group 3 proteins and mRNAs were determined separately.

RESULTS

Ten to four hundred microlitre samples of urine were collected from four mice from each sex and strain over a period of several days. The samples were dialysed individually and the protein concentration was measured after dialysis. Equal amounts (20 µg) of protein were run on IEF gels to compare the MUP components. MUPs present in different urine samples from two mice of each sex and strain are shown in Figure 3.1. Similar MUP patterns were observed in two additional mice from each sex and strain. There were minor differences in the MUP components in different urine samples from male and female C57BL/6 mice (samples from males 1

FIGURE 3.1

Isoelectric focusing gels showing several samples of urine from the following mice: Male C57BL/6, 1 and 4, female C57BL/6, 3 and 4, male Balb/c, 1 and 4 and female Balb/c, 3 and 1. Pooled urine from the same strain and sex of mice was loaded for comparison (P). Mups are labeled 1 to 5 (group 1 proteins) and 6 (group 3 proteins). Each track contained 20µg of protein and the gels were stained with coomassie blue.


♂ C57BL/6
♀ C57BL/6
P
1
4
P
3
4
6
5
4
3
2
1
♂ Balb/c
♀ Balb/c
1
4
3
1
P
6
5
4
2

and 4 and females 3 and 4 are shown in Figure 3.1), similarly, there was no difference in the MUP components in different urine samples from male Balb/c mice (samples from mouse 1 and mouse 4 are shown in Figure 3.1). In contrast, major differences in the MUP components were observed in different urine samples from all four female Balb/c mice. Urine samples from two female Balb/c mice (1 and 3) are shown in Figure 3.1.

The variation in MUP components is largely a difference in the amount of group 1 and group 3 MUPs present in each urine sample. The group 1 MUPs are MUPs 1 to 5 and the group 3 MUPs are called MUP 6. The level of MUP 6 proteins in urine from female Balb/c mice varied relative to the group 1 proteins (in this case MUP 2 and MUP 4). Additionally MUP 2 and MUP 4 are sometimes present in equal amounts and at other times there is more of MUP 4 than MUP 2. The results show that the excretion of different MUP proteins in female Balb/c varies between different urine samples.

Three parallel northern blots of liver RNA from four mice of both sexes and strains are shown in Figure 3.2. The marker tracks contained RNA transcribed *in vitro* from the group 1 cDNA clones cLiv1 and cLiv6 (Shahan *et al.*, 1987) and the group 3 cDNA clone MUP15 (Chave-Cox, 1986). An attempt was made to equalise the total amount of MUP mRNA per track by loading different amounts of liver RNA for each sex and strain (Table 3.3). The filters were re-probed with a group 1 MUP probe (Figure 3.2b) which showed that the strategy was largely successful with the main exception being two of the Balb/c females which are discussed further below.

The filters were rehybridized with an Apolipoprotein AI (ApoAI) probe to confirm the uniformity of RNA loading (Figure 3.2c). The ApoAI probe is a partial cDNA clone of Apo AI (Clissold and Bishop, 1981) which is

FIGURE 3.2

A comparison of sexually dimorphic MUP expression in the liver of C57BL/6 and Balb/c mice. To examine the effect of variable expression in individuals on sexual dimorphism, liver RNAs from four mice (1 to 4) of each sex and strain were compared. To compare the sexual dimorphism of subsets of MUP genes with total MUP and to check for evenness of RNA loading three identical northern blots were each hybridized three times (table 3.5). The first, second and third hybridizations are shown in figures 3.2a, b and c respectively. Mup expression in the liver is lower in females than males and in an attempt to equalize the total MUP RNA loaded in each track the following amounts of total liver RNA were loaded: male, 0.4µg, female C57BL/6, 3.2µg, female Balb/c, 8µg.

FIGURE 3.2a. Subsets of MUP RNAs were detected by hybridization of the three filters to the following probes; oBL1RI (top), oBS1 (middle) or group 3 (bottom). The specificity of each hybridization was confirmed by hybridization to marker RNAs; cLiv6 (top), cLiv1 (middle) or Mup15 (bottom). The numbers above marker tracks refer to ngs of RNA loaded. The MUP signal is arrowed and other bands in the top panel are due to background hybridization. The blots were autoradiographed at -70°C with screens for 3 weeks (top), 7 days (middle) or 3 days (bottom).

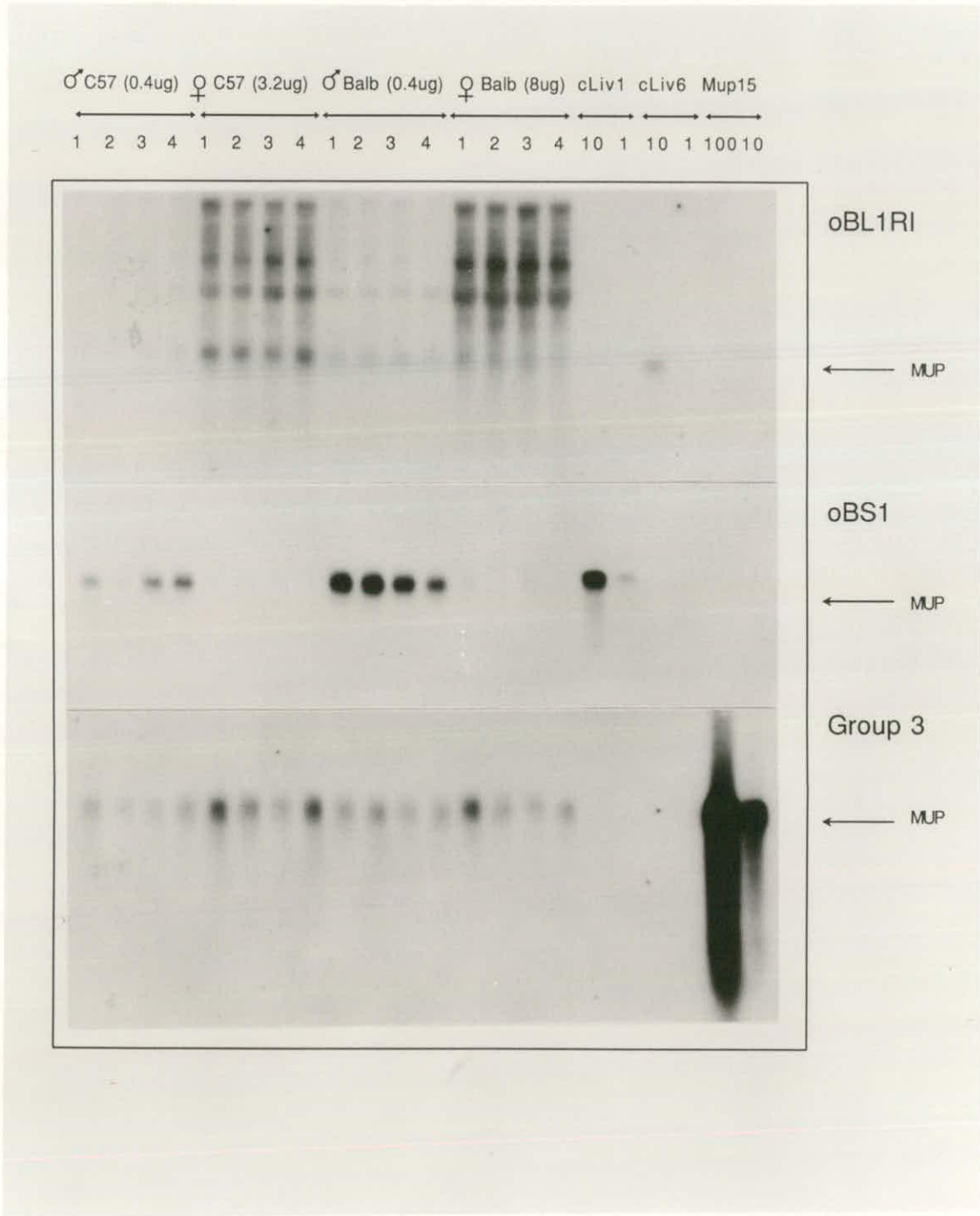

♂C57 (0.4ug)
♀C57 (3.2ug)
♂Balb (0.4ug)
♀Balb (8ug)
cLiv1
cLiv6
Mup15
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 10 1 10 1 100 10
oBL1RI
MUP
oBS1
MUP
Group 3
MUP

FIGURE 3.2b. The amount of group 1 MUP RNA in each sample was determined by rehybridizing all three blots to a group 1 probe. The bottom filter was stripped prior to rehybridization. The specificity of the hybridization was confirmed by hybridization to group 1 (cLiv1 and cLiv6) but not group 3 (Mup15) marker RNAs. The blots were autoradiographed at room temperature for 3 hours (top and middle) or 24 hours (bottom).

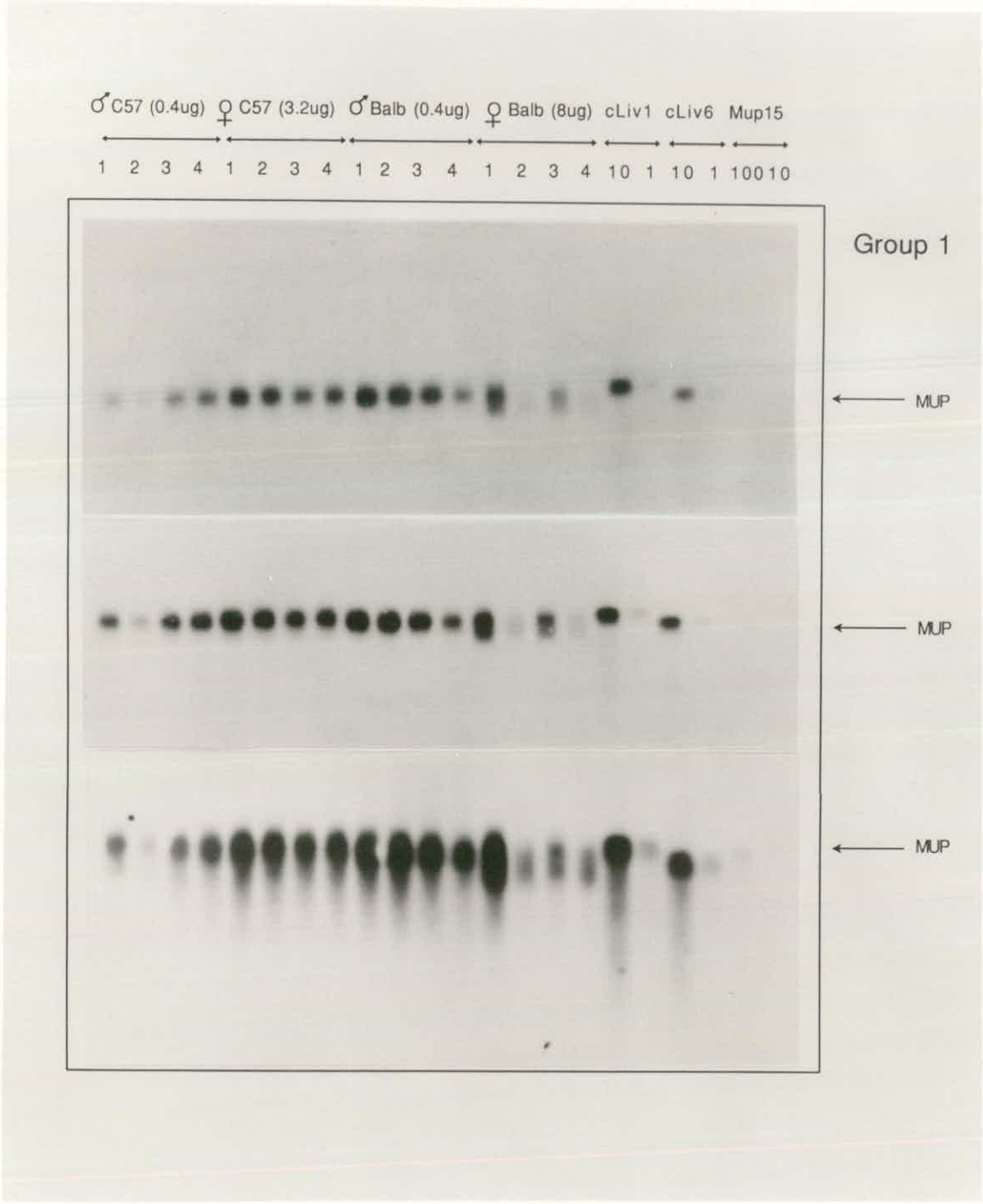

♂C57 (0.4ug)
♀C57 (3.2ug)
♂Balb (0.4ug)
♀Balb (8ug)
cLiv1
cLiv6
Mup15
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 10 1 10 1 100 10
Group 1
MUP
MUP
MUP

FIGURE 3.2c. The evenness of RNA loading was confirmed by rehybridizing the top two blots to an apolipoprotein AI cDNA probe. The blots were stripped prior to rehybridization and autoradiographed for 18 hours at -70°C with screens.

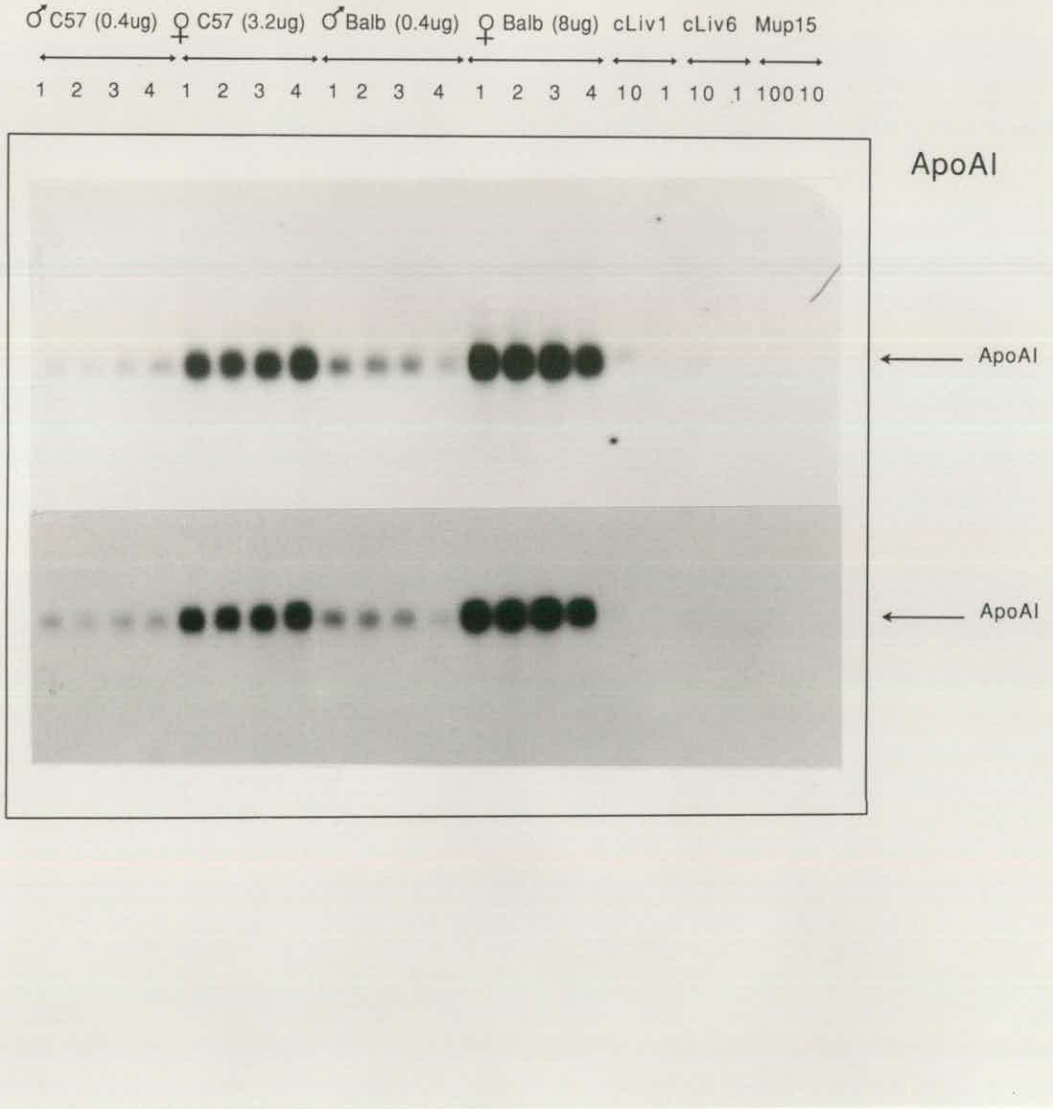

♂C57 (0.4ug)
♀C57 (3.2ug)
♂Balb (0.4ug)
♀Balb (8ug)
cLiv1
cLiv6
Mup15
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 10 1 10 1 100 10
ApoAI
ApoAI
ApoAI

TABLE 3.3 Loading of RNA

	MALE		FEMALE	
	C57	Balb/c	C57	Balb/c
RNA loaded (µg)	0.4	0.4	3.2	8
relative amount of RNA loaded	1	1	8	20

a component of plasma lipoproteins involved in lipid and cholesterol transport and is not expressed in a sexually dimorphic manner (Oscarsson *et al.* (1991). The results confirm that the same amount of RNA was loaded in the four tracks of each sex and strain. This means that the variation in MUP mRNA between the four Balb/c females (Figure 3.2b) reflects individual variation in the level of MUP mRNA relative to total RNA, rather than experimental error. McIntosh and Bishop (1989) previously reported variation in MUP mRNA levels between individual Balb/c females (Table 3.1). In contrast, the apparently low level of MUP mRNA in male C57BL/6 mice numbers 1 and 2 and male Balb/c mouse number 4 (Figure 3.2b) seems to be due to experimental error since it is matched by a low level of hybridization with the ApoAI probe (Figure 3.2c).

The sequence in which the filters were hybridized, stripped and rehybridized is shown in Table 3.4. Incomplete stripping just prior to hybridization with the ApoAI probe is assumed to be the explanation of the weak signals seen in some control tracks containing run-off MUP RNA (Figure 3.2c). The amount of group 1 probe remaining is small and does

TABLE 3.4 Sequence of hybridizations to the four filters

		FILTER		
	FIGURE	1	2	3
PROBE IN FIRST HYBRIDIZATION	3.2a	oBL1RI	oBS1	group 3 (Mup15)
STRIPPED		-	-	yes
PROBE IN SECOND HYBRIDIZATION	3.2b	GROUP1	GROUP1	GROUP1
STRIPPED		yes	yes	n.a.
PROBE IN THIRD HYBRIDIZATION	3.2c	APO AI	APO AI	-

not affect the above conclusions (compare Figures 3.2c and 3.2b).

The oligonucleotide probes oBL1RI and oBS1 were designed to hybridize to different subsets of MUP genes (Table 3.2; Chapter 1). The specificity of each oligonucleotide probe was confirmed by the its hybridization with control RNAs (Figure 3.2a). The group 3 and group 1 probes contain the 5' end of the group 3 or group 1 cDNAs Mup 15 (Chave-Cox, 1986) and cLiv1 (Shahan *et al.*, 1987a) respectively up to the EcoRI site in exon 2. The probes specifically detect group 3 or group 1 mRNA when washes are carried out under stringent conditions (Ma, 1987). The specificity of both probes was confirmed by their hybridization with group 1 or group 3 control RNA respectively (Figure 3.2b and 3.2a).

The oligonucleotide oBL1RI hybridized to RNA in liver which is the same

size as control RNA (labelled MUP; Figure 3.2a). It also hybridized to several higher molecular weight RNAs. Hybridization to these RNAs can be prevented by altering the hybridization conditions (see page 143 and Figure 4.3). This hybridization was thought to be due to non specific hybridization to ribosomal RNA. The oligonucleotide oBL1RI (Figure 3.2a) hybridizes more to the tracks containing liver RNA from C57BL/6 females than those containing liver RNA from males. This suggests that *Mup-1.1C*^{b} is expressed highly in C57BL/6 females but is a relatively minor component in C57BL/6 males. Therefore the sexual dimorphism of *Mup-1.1C*^{b} RNA is lower than total MUP. The oligonucleotide oBL1RI hybridizes equally to the tracks containing male and female Balb/c liver RNA. This suggests that the expression of *Mup-1.1C*^{a} in common with total MUP is twenty times higher in male than female Balb/c liver. This agrees with McIntosh and Bishop (1989) who examined the expression of *Mup-1.1C*^{a} in Balb/c mice with the oligonucleotide oBL1 and observed male/female ratios of 7, 13, 14 and 100. Using the above results to compare the sexual dimorphism of *Mup-1.1C*^{b} and *Mup-1.1C*^{a} suggests that *Mup-1.1C*^{a} expression is approximately ten times more sexually dimorphic than *Mup-1.1C*^{b} expression.

oBS1 hybridized more strongly to the tracks containing male liver RNA from both strains than tracks containing female liver RNA (Figure 3.2a). Taking account of the different amounts of RNA loaded, the sexual dimorphism must be in the order of 100 which is considerably higher than the mean for all MUPs. This is in complete agreement with the results obtained with Balb/c mice by McIntosh and Bishop (1989).

In summary, these results show that different group 1 genes are subject to different regulation. The sexual dimorphic expression of *Mup-1.1C*^{b} is in the order of 2, that of the allelic gene *Mup-1.1C*^{a} is between 7 and 20. mRNA which hybridizes with oBS1 has a total sexual dimorphism

of approximately 100 in both strains of mice.

There is considerable divergence between the group 1 and group 3 genes. The two families respond differently to hormonal treatment and group 3 proteins are N-glycosylated. In contrast to the levels of group 1 RNA which were consistent in all four C56BL/6 females (Figure 3.2b), the level of group 3 RNA was higher in females 1 and 4 (Figure 3.2a). The levels of both group 1 and group 3 RNA varied between individual Balb/c females (Figure 3.2a and 3.2b) but the variation in group 3 RNA was lower than group 1 RNA. Hence, group 3 and group 1 RNA make up varying proportions of total MUP RNA in different females but always make up the same proportions in different males. This differs from the protein data where it was shown that group 1 and group 3 proteins were excreted equally by C57BL/6 females and males and Balb/c males but excreted at different levels by Balb/c females.

Clark *et al.* (1985) and Chave-Cox (1986) identified two different size classes of MUP mRNA, long and short. Short mRNA contains exons 1 to 6, whereas long message contains a shorter 5' region of exon 6 (6a) and exon 7. The short RNA has two polyadenylation signals, both of which are used (Clark *et al.*, 1984) in addition some long mRNAs contain a longer version of exon 6a (Ma, 1987; Shahan *et al.*, 1987a). An equal amount of two different sizes of group 1 mRNA were present in female Balb/c liver RNA whereas a much higher amount of long than short message was present in male Balb/c and male and female C57BL/6 liver (Figure 3.2b). Out of three *Mup-1.1C*[2] cDNA clones, two are spliced as the long transcript (Shahan *et al.*, 1987a; Chave-Cox, 1986), and one is spliced as the short transcript (Clissold and Bishop, 1981; Chave-Cox, 1986). It is unlikely that this is a difference in splicing peculiar to Balb/c females because the short cDNA clone was isolated from a Balb/c male and the oligonucleotide oBL1RI hybridizes more to

the long than the short message in the Balb/c female tracks (compare Figures 3.2a&b). It is more likely that a group 1 gene which is expressed at a relatively high level in Balb/c females is spliced to generate greater amounts of short message.

DISCUSSION

In female Balb/c urine the group 1 and group 3 proteins are not always present in the same proportions. The proportions vary in different urine samples from the same mouse. A similar effect is observed in the liver mRNA population where levels of group 3 message in different mice are much more uniform than the levels of group 1 message. It is not clear whether variation in the level of group 1 message is due to a difference between mice or reflects temporal fluctuations within the mice. However, the results obtained from studying MUPs in urine suggest that some variation occurs within each mouse. Variation may therefore be due to hormonal rather than genetic factors. The four mice from each sex and strain were housed together and killed at the same time of day, similarly the urine samples were taken at the same time of day on successive days. This suggests that the variation does not have a diurnal rhythm. Since the variation occurs in female mice it may relate to the ovarian cycle. This could be investigated.

The results do not rule out the possibility that the variation in liver RNA and urinary protein are two unrelated observations. The variation in liver RNA levels may be due to variable expression of MUP genes in different mice due to some epigenetic factors like DNA methylation. The variation in excretion may be due to factors which affect excretion of group 1 and group 3 MUPs differently. The group 3 MUPs are known to be glycosylated whereas the group 1 MUPs are not which could lead to

differences in their excretion. Differences might occur in the secretory pathway in hepatocytes, in plasma transport, or in the rates of glomerular secretion, tubular uptake or extracellular degradation in the kidneys. In order to compare group 1 MUP expression in individual Balb/c female mice it might be better to compare their total MUP excretion over several days with their liver RNA levels.

Mup-1.1C^{a} and *Mup-1.1C*^{b} mRNAs are present in male and female liver RNA from either Balb/c or C57BL/6 mice respectively, consistent with the expression of their protein products (Chapter 2). In female C57BL/6 liver RNA *Mup-1.1C*^{b} mRNA was the major MUP message which agrees with the observation that the protein specified by *Mup-1.1C*^{b} is the major protein in female C57BL/6 urine (Chapter 2). In female Balb/c mice there are at least two predominant messages. One was *Mup-1.1C*^{a} RNA which agrees with the presence of the protein specified by this gene as one of two major proteins in female Balb/c urine (Chapter 2).

Another group 1 gene expressed in female Balb/c liver may have the same sequence as the group 1 genomic clone BS6. Three pieces of evidence are consistent with this. 1) Chave-Cox (1986) sequenced two different group 1 cDNA clones isolated from a female Balb/c liver cDNA library. One (Mup8) was a partial cDNA clone of *Mup-1.1C*^{a} and the other (Mup11) had the same coding sequence as the genomic clone BS6. 2) Two sizes of group 1 MUP mRNA, long and short, were present in RNA from female Balb/c liver. Hybridization with specific oligonucleotide probes showed that *Mup-1.1C*^{a} mRNA is spliced mainly as the long type. Thus another group 1 MUP mRNA is spliced to generate the short mRNA. A MUP-hybrid transgene containing the 3' region of BS6 including exons 6 and 7, produced an equal amount of long and short mRNA in the liver. This indicates that the 3' sequences of BS6 can be spliced to generate both types of mRNA (Al-Shawi *et al.*, (submitted)). 3) The protein specified

by BS6 is one of the two major proteins in female Balb/c liver (Chapter 2).

TABLE 3.5 Sexual dimorphism of MUP expression

strain	mRNA in liver (copies / cell)		reference
	male	female	
Balb/c	30,000	6,150	Hastie *et al.*, 1979
C57BL/6	30,000	6,200	Knopf *et al.*, 1983

The levels of all MUP mRNAs were found to be lower in Balb/c than C57BL/6 females but similar in Balb/c and C57BL/6 males. An estimation of the sexual dimorphism of MUP mRNA in C57BL/6 liver is 8:1 compared with 20:1 in Balb/c liver. The levels of MUP mRNA were previously shown to be approximately equal in both male and female Balb/c and C57BL/6 livers (Table 3.5). The difference between these results may be due to different mouse colonies or it may be due to the variation between individual Balb/c females.

Consistent with the expression of total group 1 mRNA in female mice, the expression of *Mup-1.1C*^{b} in Balb/c females was found to be lower than that of *Mup-1.1C*^{a} in C57BL/6 females. In contrast the levels were approximately equal in males. As mentioned in the introduction the difference in the expression of MUP genes may be due to differences in *cis*-acting regulatory sequences present in the individual genes, the

MUP loci or to differences in *trans*-acting modifiers present in the two strains. MUP expression in female mice of different species varies from none to equal with males (Sampsell and Held, 1985). *Trans*-acting modifiers might have been introduced from matings with these species during the isolation of inbred strains (Watanabe *et al.*, 1989; Nobuhara *et al.*, 1989). There are several *trans*-acting loci in inbred strains of mice which affect the level of MUP excretion in females (Berger and Szoka, 1981; Duncan *et al.*, 1988). Several *cis*-acting sequences are also present in MUP alleles. A *cis*-acting factor in the wild mouse species *M. spretus* causes the *M. spretus* MUPs to be expressed at low levels in females (Sampsell and Held, 1985).

It should be possible to determine if factors which cause lower expression of group 1 genes in Balb/c females compared with C57BL/6 females act in *cis* or in *trans* by studying the expression of group 1 genes in female Balb/c X C57BL/6 F_1s. mRNA from the Balb/c gene *Mup-1.1C*^{a} could be distinguished by hybridizing with oBL1 (Chapter 1). mRNA from both *Mup-1.1C*^{a} and *Mup-1.1C*^{b} can be detected by hybridizing with oBL1RI. The hybridizations with oBL1 and oBL1RI could be compared to determine the relative expression of *Mup-1.1C*^{b} and *Mup-1.1C*^{a}. It should also be possible to determine whether the factors causing variation in group 1 expression between individual Balb/c females is transmitted in *cis* or in *trans* by comparing expression of *Mup-1.1C*^{b} and *Mup-1.1C*^{a} in a number of Balb/c X C57BL/6 F_1 females.

The linkage group and possible identity of *trans*-acting modifiers could be determined by studying *Mup-1.1C*^{a} and *Mup-1.1C*^{b} expression in Balb/c X C57BL/6 recombinant inbred strains. In order to determine the identity of any *cis*-acting sequences, clones of the *Mup-1.1C* alleles could be utilized to make transgenes. Several constructs containing part or all of a number of MUP clones have already been utilized to

make transgenes which were correctly regulated (Shi *et al.*, 1989; Al-Shawi *et al.*, 1988; Held *et al.*, 1989). In order to distinguish mRNA from the MUP transgene from the endogenous MUP mRNA single or multiple mutations could be introduced into the transgene by site directed mutagenesis. If the important *cis*-acting sequences are present in the MUP clones then the expression of the transgene would be similar to the endogenous gene. Hybrid genes could then be made between the *Mup-1.1C*^{b} and *Mup-1.1C*^{a} clones which would allow different regions of each clone to be compared and thus determine which regions are important for the different patterns of expression.

The oligonucleotide oBS1 hybridizes to five different group 1 genes in Balb/c and C57BL/6 mice (Chapter 1). In agreement with previous studies on Balb/c mice (McIntosh and Bishop, 1989) the RNA which hybridizes to oBS1 is found almost exclusively in males which is consistent with the expression of proteins specified by genes in the *Mup-1.1B* subfamily in male but not female liver (Table 2.5). The genes which hybridize to oBS1 and are expressed, are highly sexually dimorphic. The presence of only two proteins in C57BL/6 and one in Balb/c which are very sexually dimorphic (Chapter 2) is consistent with the idea that many of the genes which hybridize with oBS1 may be silent (Shi *et al.*, 1989). *Mup-1.1B* genomic clones could be utilized to make transgenes and the pattern of expression of these should be different to that expected for *Mup-1.1C* transgenes. These could also be used as above to make hybrid MUP genes and the regions which determine the highly sexually dimorphic expression of these genes could be identified.

CHAPTER 4

THE EXPRESSION OF MUP GENES IN THE LIVER OF GROWTH HORMONE DEFICIENT MICE

In normal rats and mice, GH secretion by the pituitary is pulsatile and the frequency of the pulsation is sex dependent. In female rats and mice the pulses of GH secretion are more frequent than in males. In female rats the short pulse interval leads to a level of GH in the serum which is almost continuous whereas in male rats a pulse of release is followed by a period when circulating GH is almost undetectable (Jansson *et al.*, 1985). The liver is able to distinguish between pulsatile and continuous plasma GH and in response produces two distinct patterns of gene expression, masculine and feminine, involving the expression of different subsets of liver genes (Jansson *et al.*, 1988; Noshiro and Negishi, 1986; MacLeod and Shapiro, 1989).

GH deficiency in mice or rats can be caused by genetic mutations affecting GH release (Beamer and Eicher, 1976; Schaible and Gowen, 1961; Bartke, 1964; Camper *et al.*, 1990; Takeuchi *et al.*, 1990; Charlton *et al.*, 1988), genetic ablation of the somatotrophs (Borrelli *et al.*, 1989; Behringer *et al.*, 1988) or by surgical removal of the hypophysis. In GH deficient mice or rats, the sexually dimorphic expression of GH responsive genes in the liver is lost. To restore sexually dimorphic gene expression, GH must be administered to mimic the pulsatile male or more continuous female pattern (Norstedt and Palmiter, 1984; Shapiro *et al.*, 1989; Noshiro and Negishi, 1986; McLellan-Green *et al.*, 1989; Oscarsson *et al.*, 1991; Jansson *et al.*, 1988; Kato *et al.*, 1986; Waxman *et al.*, 1989; Robertson

et al., 1990).

"Little" (*lit/ lit*) mice are GH deficient mutants maintained on a C57BL/6 background (Beamer and Eicher, 1976). *lit/lit* mice are deficient in GH but not in other pituitary hormones (prolactin, thyrotropin, luteinizing hormone or follicle stimulating hormone) (Phillips *et al.*, 1982) or thyroxine (Chubb and Henry, 1987). *Lit/lit* mice have 8% of the GH mRNA and 5 to 10 % of the circulating GH found in wild type mice (Cheng *et al.*, 1983; Phillips *et al.*, 1982) and are unable to release GH in response to GRF, which is consistent with a mutation in the GRF receptor (Jansson *et al.*, 1986).

The homozygous *lit* mutation produces a number of phenotypic effects, including a loss of GH dependent growth and the sexually dimorphic expression of several genes in the liver (Eicher and Beamer, 1979; Jansson *et al.*, 1988; Norstedt and Palmiter, 1984; Noshiro and Negishi, 1986). *Lit/lit* mice grow normally when treated with exogenous GH (Beamer *et al.*, 1976). The numbers and binding characteristics of GH receptors in the liver of *lit/lit* mice are similar to normal mice (Herington *et al.*, 1983) and if GH is administered to mimic the secretory profiles of normal males or females, sexually dimorphic gene expression is induced in the liver (Norstedt and Palmiter, 1984; Noshiro and Negishi, 1986).

As discussed, the pattern of MUPs in the urine and the level of MUP mRNA in the liver is normally sexually dimorphic with expression being higher in males and the females expressing a subset of the male proteins at a lower level. In contrast to the normal expression, both male and female *lit/lit* mice have very low levels of MUP mRNA in the liver and MUPs in the urine (Norstedt and Palmiter, 1984). Pulsatile administration of GH to

lit/lit mice to imitate the plasma levels in normal males raised both MUP mRNA in the liver and MUP in urine to the male level. In contrast continuous administration of the same dose of GH failed to significantly induce MUP mRNA or MUP in the urine. This level of GH administration was effective in inducing female specific expression since continuous administration of the same dose of GH to normal male mice interrupted their normal pulsatile plasma profile of GH and consequentially induced female-like numbers of PRL-Rs in liver membranes (Norstedt and Palmiter, 1984).

Several MUP genes are known to be expressed in the male liver and only a subset of these are expressed in the female liver (Knopf *et al.*, 1983). Measuring total MUP mRNA in the liver does not allow a distinction to be made between these two patterns of MUP expression (Norstedt and Palmiter, 1984). The level of MUP mRNA in *lit/lit* liver is much lower than in normal liver, however it is not known whether MUP expression is still sexually dimorphic in pattern (Norstedt and Palmiter, 1984). This is important because low levels of GH in plasma can induce sexually dimorphic expression of cytochrome P450s in the liver (Waxman *et al.*, 1991) which probably accounts for the sexually dimorphic pattern of gene expression observed in the dwarf rats (Bullock *et al.*, 1991).

By examining MUP proteins from *lit/lit* urine by IEF it is possible to determine whether the pattern of MUP expression is sexually dimorphic in *lit/lit* mice. The oligonucleotides described here (Chapters 1 and 3) can allow subsets of MUP genes with different patterns of sexually dimorphic expression in the liver to be examined. The oligonucleotide oBS1 hybridizes to a subset of MUP mRNAs which are present in male but not female C57BL/6 liver, whereas the oligonucleotide oBL1RI hybridizes to a

single MUP mRNA which is present in both male and female liver (Chapter 3). The expression of MUPs was examined in *lit/+* and *lit/lit* mice and was found to be sexually dimorphic in *lit/+* but not *lit/lit* mice. GH was administered to male *lit/lit* mice by two different methods to imitate normal male or female GH secretion and this induced the MUP genes normally expressed in male or female *lit/+* livers respectively.

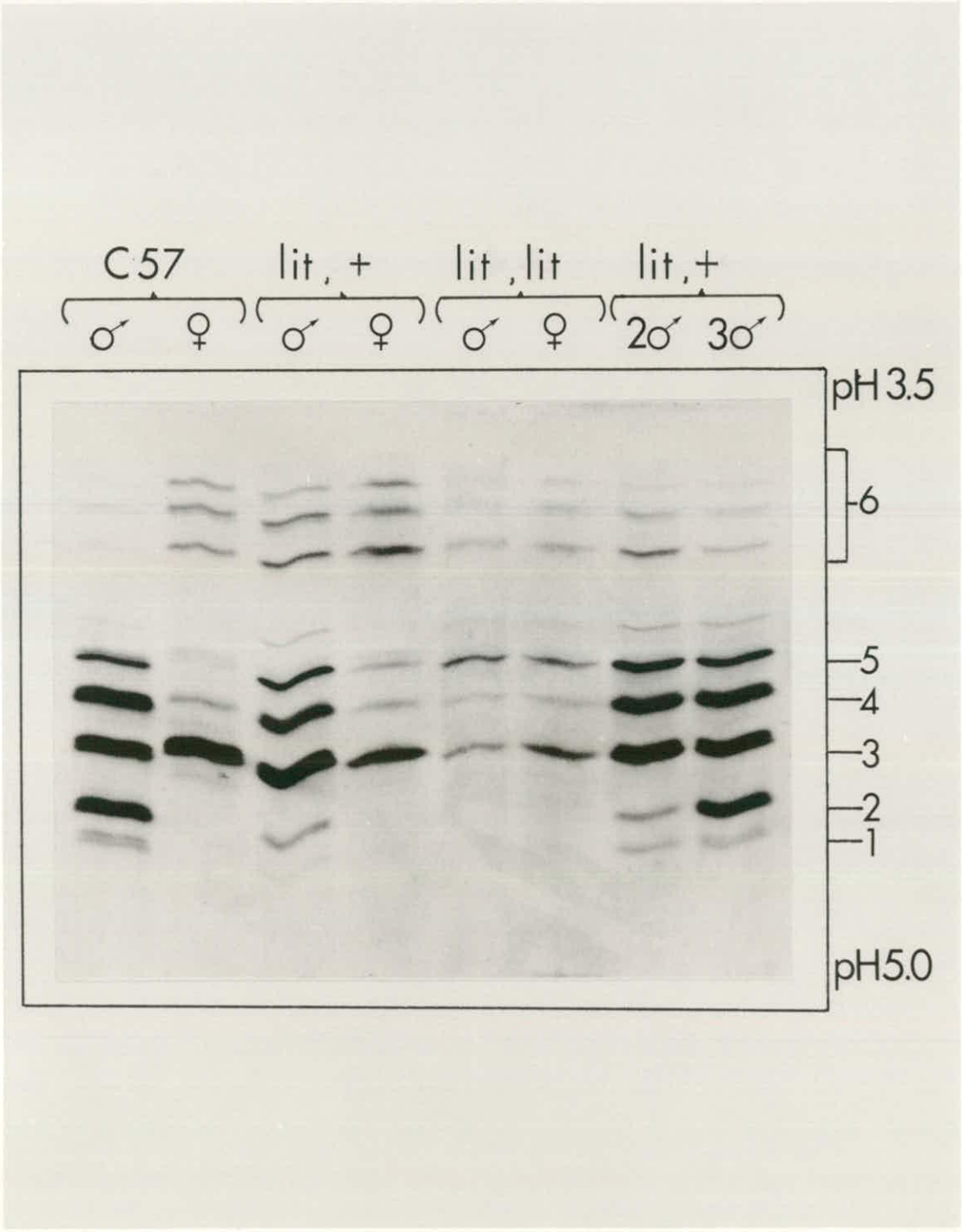
RESULTS

Urine was collected from mutant *lit/lit* mice, heterozygous but phenotypically normal *lit/+*^{l} mice and normal +^{b}/+^{b} mice. +^{l} indicates a mouse from the *lit/lit* colony whereas +^{b} indicates a C57BL/6 mouse from Banton and Kingman. These lines of mice have been bred separately for at least seven years. Urine from several mice was pooled and dialysed, then the protein concentration was measured. In female +^{b}/+^{b} and *lit/+*^{l} urine the protein concentration was approximately 25% of the concentration in male +^{b}/+^{b} and *lit/+*^{l} urine. In both male and female *lit/lit* urine the protein concentration was approximately 10% of the concentration in male +^{b}/+^{b} urine.

In order to analyse the MUP components present in normal and mutant urine 20µg samples of urine from +^{b}/+^{b}, *lit/+*^{l} and *lit/lit* male and female mice were analysed by means of IEF gels (Figure 4.1). The total amount of MUP present in 20µg of protein from *lit/lit* urine is lower than from +^{b}/+^{b} or *lit/+*^{l} urine because there are other non-MUP proteins present in urine which can be detected by SDS PAGE (data not shown) and make up a higher proportion of protein in *lit/lit* urine than they do in +^{b}/+^{b} or *lit/+*^{l} urine. In urine from normal +^{b}/+^{b} mice and heterozygous *lit/+*^{l} mice, the MUP proteins are sexually dimorphic (Figure 4.1). The pattern of MUPs in

FIGURE 4.1

MUP proteins in urine from male and female C57BL/6, *lit/+* and *lit/lit* mice and two individual male *lit/+* mice were compared by isoelectric focusing. 20µg of protein was loaded in each track and the gel was stained with coomassie blue.


C57
lit, +
lit, lit
lit, +
♂
♀
♂
♀
♂
♀
2♂
3♂
pH 3.5
6
5
4
3
2
1
pH 5.0

lit/lit urine is different from either $+^{b}/+^{b}$ or *lit*/$+^{l}$ male or female patterns and is not sexually dimorphic. *lit/lit* mice do not produce MUPs 1 and 2 which are found in urine from male $+^{b}/+^{b}$ and *lit*/$+^{l}$ mice. The *lit/lit* urine does contain MUPs 3, 4, 5 and 6 which are present in both male and female $+^{b}/+^{b}$ and *lit*/$+^{l}$ urine. However the relative levels of the different MUPs are different from those in female *lit*/$+^{l}$ or $+^{b}/+^{b}$ urine (Figure 4.1). In summary, in *lit/lit* mice there is no sexual dimorphism in either the level and pattern of MUPs in urine.

The MUPs present in urine from male and female $+^{b}/+^{b}$ mice differed from those present in urine from male and female *lit*/$+^{l}$ mice. In male *lit*/$+^{l}$ urine there was a lower level of MUPs 1 and 2 than in male $+^{b}/+^{b}$ urine. Presumably this relates to the absence of MUPs 1 and 2 in male *lit/lit* urine. It therefore seems possible that the phenotype of male *lit*/$+^{l}$ mice is intermediate between the male *lit/lit* pattern and the male $+^{b}/+^{b}$ pattern. A similar effect was seen in female *lit*/$+^{l}$ mice. The level of MUP 5 was higher in female *lit/lit* urine than $+^{b}/+^{b}$ urine and the level in *lit*/$+^{l}$ urine was intermediate between the two. Also MUP 3, the major protein in female $+^{b}/+^{b}$ urine was reduced in female *lit/lit* urine and the level in *lit*/$+^{l}$ was intermediate. Thus, the pattern of MUP secretion in male and female *lit*/$+^{l}$ urine is intermediate between the patterns in male and female $+^{b}/+^{b}$ and *lit/lit* urine respectively.

Urine from two individual *lit*/$+^{l}$ males was examined (Tracks 7 and 8, Figure 4.1). The proteins in the urine from these two individual *lit*/$+^{l}$ mice were different. Proteins from one mouse were the same as those in pooled *lit*/$+^{l}$ urine, whereas those from the other mouse were the same as those in pooled $+^{b}/+^{b}$ urine. Therefore the difference between pooled *lit*/$+^{l}$ and $+^{b}/+^{b}$ urine is not due to the *lit* mutation but to variation amongst

the *lit/+* mice.

TABLE 4.1 Loading of RNA

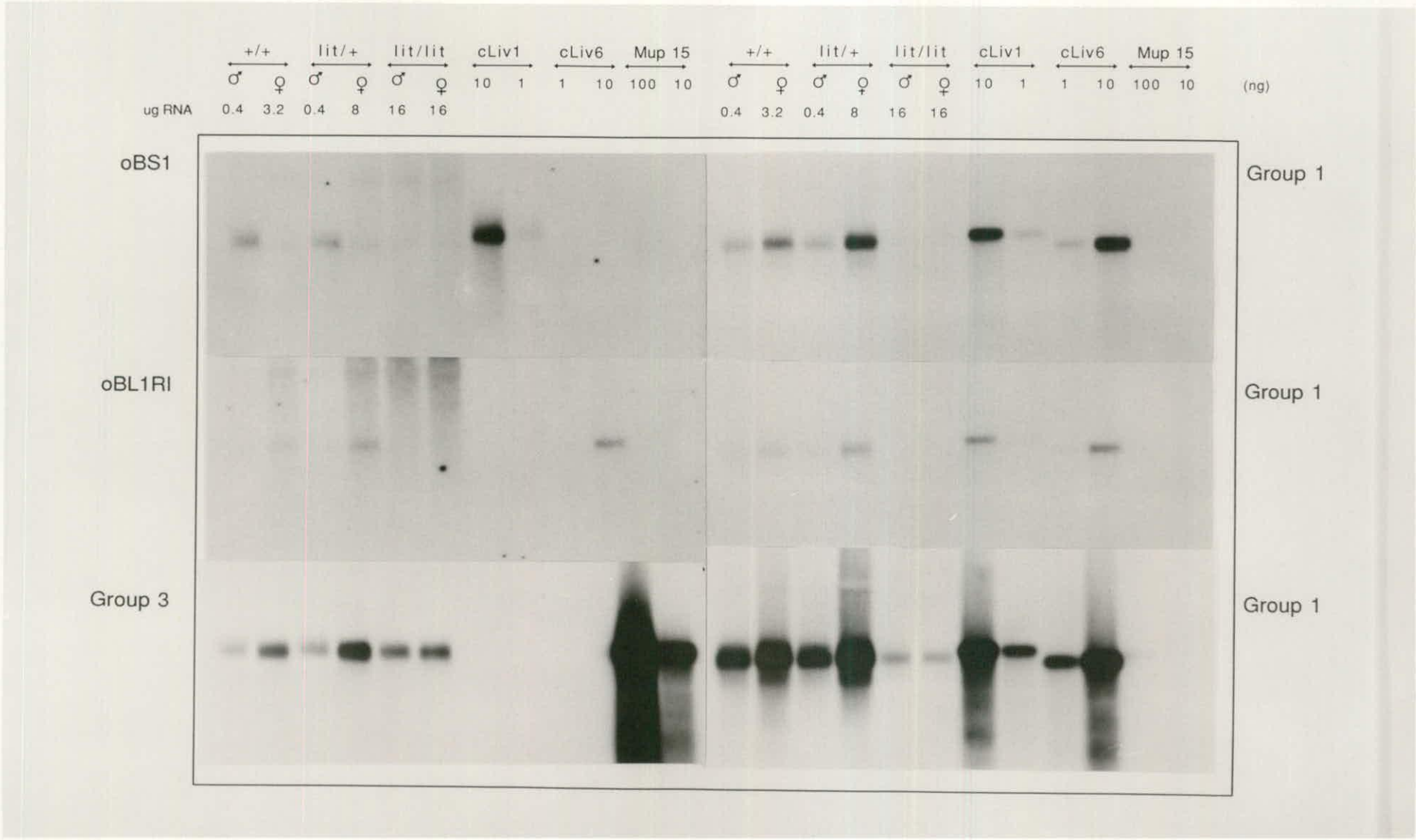
	MALE		FEMALE		
	C57, *lit/+*^{1}	*lit/lit*	C57	*lit/+*^{1}	*lit/lit*
RNA loaded (ug)	0.4	16	3.2	8	16
relative amount	1	40	8	20	40

Two parallel northern blots were prepared containing RNA from male and female +^{b}/+^{b}, *lit/+*^{1} and *lit/lit* mice. Again different amounts of RNA were loaded in each track in an attempt to equalize the loading of total MUP RNA per track. This was only partially successful as judged by hybridization to a group 1 probe (Figure 4.2 RHS). The RNA loading is shown in Table 4.1.

The Northern blots were each hybridized twice. Blot 1 was hybridized first with oBS1 and then with a group 1 probe. This filter was exposed differently on three occasions and the photographs of all three films are shown (Figure 4.2, RHS). Blot 2 was hybridized first with oBL1RI and then

FIGURE 4.2

A comparison of the sexual dimorphism of MUP expression in the liver of individual $+^b/+^b$, *lit*/$+^b$ and *lit/lit* mice. Subsets of MUP RNAs were detected by hybridization of two identical filters to oBS1 (top) and oBL1RI (middle) and then rehybridizing one filter to group 3 (bottom) and the other to group 1 (right hand panel, three different exposures). The specificity of each hybridization was confirmed by hybridization to marker RNAs; cLiv1 (top), cLiv6 (middle), Mup15 (bottom) or cLiv1 and cLiv6 (Right hand side). Blots were autoradiographed at -70°C with screens for 3 weeks (oBS1 and oBL1RI) or 24 hours (group 3) or at room temperature (group 1) for 1 hour (top), 3 hours (middle) or 24 hours (bottom). Amounts of total liver RNA were loaded are indicated above the tracks. The numbers above marker tracks refer to ng of RNA loaded.


+/+
lit/+
lit/lit
cLiv1
cLiv6
Mup 15
♂ ♀ ♂ ♀ ♂ ♀
10 1 1 10 100 10
(ng)
ug RNA
0.4 3.2 0.4 8 16 16
0.4 3.2 0.4 8 16 16
oBS1
oBL1RI
Group 3
Group 1
Group 1
Group 1

a group 3 probe (Figure 4.2). The specificity of each probe was judged by its ability to distinguish between homologous and non homologous RNA markers (Chapter 3). As a result of the differential loading of RNA the group 1, group 3 or oBL1RI probes hybridized more to tracks containing female than male $+^{b}/+^{b}$ and *lit*/$+^{l}$ RNA, however oBS1 hybridized more to tracks containing male than female RNA indicating that the sexual dimorphism of MUP expression is similar in *lit*/$+^{l}$ and $+^{b}/+^{b}$ mice.

In contrast all four probes hybridized equally to tracks containing male and female *lit/lit* RNA indicating that there is no sexual dimorphism in MUP expression in *lit/lit* mice. RNA from the *lit/lit* mice hybridized very poorly with the group 1 probe. Compared with the ten-fold ratio in protein present in urine, the signal is disproportionately low relative to a $+^{b}/+^{b}$ male (for example). This is partly accounted for by a higher production of group 3 MUP (Figure 4.1) due presumably to the higher production of group 3 MUP mRNA observed (Figure 4.2). The presence of non-MUP proteins in the urine probably also contributes to the apparent discrepancy.

GH is capable of inducing MUP expression in *lit/lit* mice (Norstedt and Palmiter, 1984). The pattern of GH administration determines the level of MUP expression. Pulsatile delivery of GH by injection to *lit/lit* mice increases MUP mRNA to normal male *lit*/$+^{l}$ levels, whereas continuous delivery of GH by minipump caused an insignificant increase in MUP mRNA (Norstedt and Palmiter, 1984), but is capable of inducing PRL-R expressions to levels normally found in females.

Experiments described here show that MUP mRNA present in male *lit*/$+^{l}$ mice can hybridize to both oBS1 and oBL1RI whereas MUP mRNA present in female *lit*/$+^{l}$ mice hybridizes to oBL1RI but not oBS1. Neither type of mRNA are

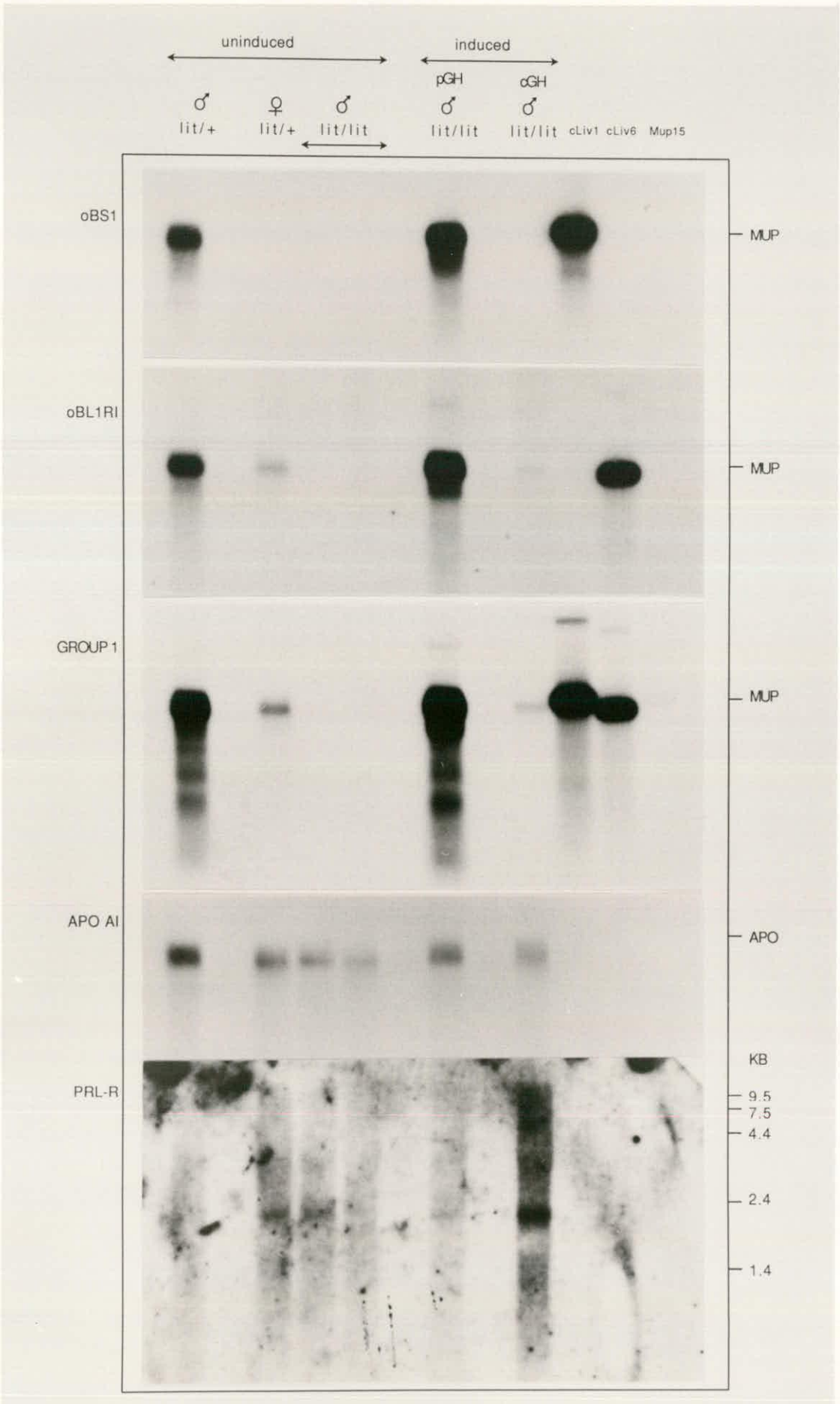
present in *lit/lit* mice which implies that pulsatile GH can induce both types of MUP mRNA but continuous GH is unable to induce MUP mRNA homologous to oBS1.

GH was administered to male *lit/lit* mice in either a pulsatile manner to mimic the normal male plasma pattern of GH or a continuous manner to mimic the normal female pattern. MUP mRNA levels in the liver were analysed by northern blots (Figure 4.3). The two regimes of GH administration had different effects on MUP mRNA in male *lit/lit* mice. The levels of MUP mRNA in *lit/lit* males induced with pulsatile bGH were similar to those found in *lit/+*^{1} males. The levels of MUP mRNA in *lit/lit* males induced with continuous oGH were similar to but slightly lower than those found in *lit/+*^{1} females (Figure 4.3 (This effect is underestimated in Figure 4.3 due to the low expression of MUP in the female *lit/+*^{1} mouse shown. This mouse had lower levels of MUP mRNA than the *lit/+*^{1} female shown in Figure 4.2 which may indicate variation in expression between individual *lit/+*^{1} females)). Rehybridization of the blot to an ApoAI probe showed that this was not due to uneven loading of RNA (Figure 4.3).

The induction of MUPs by pulsatile bGH occurs over a period of days (data not shown). The pulsatile bGH was administered for five days whereas the continuous oGH was administered for three days which may have contributed to the difference in the level of induction by these two types of hormone treatment. The dose of continuous oGH administered was capable of over-inducing PRL-R mRNA compared with *lit/+*^{1} females (Figure 4.3) which indicates that this reasoning does not apply for all GH induced liver RNAs. In addition different MUP mRNAs were induced by the two different regimes of GH administration. mRNA from male *lit/lit* mice induced with pulsatile bGH hybridized to both oBS1 and oBL1RI. The pattern of hybridiza-

FIGURE 4.3

A comparison of the sexual dimorphism of MUP expression in the liver of individual *lit/+*[1] mice and the effect of different patterns of GH administration on the pattern of MUP expression in male *lit/lit* mice. Uninduced male *lit/lit* mice were treated with buffer. Induced *lit/lit* mice were treated with pulsatile GH (pGH) or continuous GH (cGH). Subsets of MUP RNAs were detected by hybridization of two identical filters to oBS1 and oBL1RI and rehybridizing one filter to group 1. The specificity of each hybridization was confirmed by hybridization to marker RNAs; cLiv1 and cLiv6. The effectiveness of continuous GH on gene expression in the liver was confirmed by rehybridizing both blots with a PRL-R cDNA probe (bottom panel). The size markers (BRL RNA ladder) indicated on the right of this gel showed that size of the major PRL-R message was approximately 2.0kb. The evenness of RNA loading was confirmed by rehybridizing with an apolipoprotein AI cDNA probe (Apo AI). 30µg of RNA was loaded per track or 30ng per marker track. Blots were autoradiographed at -70°C with screens for 3 hours (ApoAI), 5 hours (oBS1), 3 days (oBL1RI), 7 days (PRL-R) or at room temperature for 10 hours (group 1).


uninduced
induced
pGH
oGH
♂ lit/+
♀ lit/+
♂ lit/lit
♂ lit/lit
♂ lit/lit
cLiv1
cLiv6
Mup15
oBS1
oBL1RI
GROUP 1
APO AI
PRL-R
MUP
MUP
MUP
APO
KB
9.5
7.5
4.4
2.4
1.4

tion was similar to *lit/+*^{1} males (compare tracks 1 and 5, Figure 4.3). RNA from male *lit/lit* mice induced with continuous oGH did not hybridize to oBS1 but did hybridize to oBL1RI and the pattern of hybridization was similar to normal *lit/+*^{1} females (compare tracks 2 and 6, Figure 4.3).

DISCUSSION

In contrast to the situation in *lit/+* mice, the expression of group 3 genes in *lit/lit* mice was found to be higher than group 1 genes, which indicates that group 3 genes are less dependent on GH. This agrees with the ten to twenty fold reduction of group 3 mRNA and the several hundred fold reduction in group 1 RNA observed in hypophysectomized mice (Kuhn *et al.*, 1984).

Specific oligonucleotide probes were used to study the expression of subsets of group 1 genes in the liver which are regulated differently by GH. *Mup-1.1C*^{b} is detected with the oligonucleotide oBL1RI. It is expressed in both male than female *lit/+* liver although expression is higher in males. It is not expressed in the liver of *lit/lit* mice of either sex. In contrast, MUP genes which hybridize to the oligonucleotide oBS1 are only expressed in male *lit/+* liver and not in livers of female *lit/+* or *lit/lit* mice of either sex. Two regimes of GH administration led to an increase in *Mup-1.1C*^{b} expression in male *lit/lit* mice; treatment with bGH by injection for five days induced much higher levels of expression than treatment with oGH by infusion for three days. In contrast, only one regime, treatment with bGH by injection for five days, induced MUP RNA which hybridized with oBS1. Thus, the two subsets of group 1 MUP genes, *Mup-1.1C*^{b} and genes which hybridize to oBS1 respond differently to GH. As

the patterns of plasma GH are different in male and female mice (MacLeod *et al.*, 1991) different levels of sexually dimorphic expression of these two subsets of group 1 MUP genes could be due to differences in their response to endogenous GH.

PRL-R mRNA is present at higher levels in the livers of female *lit/+* mice than male *lit/+* or *lit/lit* mice of either sex which is consistent with the levels of PRL-R in liver membranes (Norstedt and Palmiter, 1984). The two regimes of GH treatment had inverse effects on PRL-R mRNA and specific MUP mRNAs. Treatment with oGH by infusion for three days led to higher levels of PRL-R mRNA than treatment with bGH by injection for five days. This is consistent with levels of induction of total MUP mRNA in the liver and PRL-Rs in liver membranes by different regimes of bGH administration (Norstedt and Palmiter, 1984).

MATERIALS AND METHODS

ISOLATION OF GENOMIC DNA

DNA was isolated from liver by the method of Birch and Weintraub (1986). The tissue was rinsed in an isotonic buffer (0.15M NaCl, 0.015M NaCitrate, 10mM Tris Cl pH 7.4). It was homogenized by Dounce homogenization in 10mM Tris-Cl, 10mM NaCl, 3mM $MgCl_2$ pH 7.4, 0.5% NP40. The homogenate was filtered through 100µm gauze and the nuclei were isolated by centrifugation at 9,000 rpm at 4°C for 5 minutes. The nuclei were resuspended in the same buffer and recentrifuged three times to obtain clean nuclei. The nuclei were digested with 100µg/ml protease K and 2µg/ml RNaseA in 1% SDS, 5mM NaCl, 85mM Tris pH 7.6, 80mM EDTA at 65°C for 1 hour. Nuclear DNA was extracted once with a 1:1 mixture of phenol (equilibrated with 1M Tris-Cl pH 8.0) and chloroform and twice with chloroform alone. It was dialysed at 4°C overnight against 100mM NaCl, 10mM Tris-Cl pH 7.6, 1mM EDTA, then overnight against 10mM Tris pH 7.6, 1mM EDTA. The DNA concentration was measured by reading the absorbance of an aliquot of the DNA at 260nm. The DNA was stored at 4 to 8°C. The length was assessed by comparing it to bacteriophage lambda DNA on a 0.4% Tris-borate agarose gel.

DIGESTION OF GENOMIC DNA

Digestions were done using the buffers and conditions supplied by the manufacturer. In problem digests the DNA was incubated with spermidine (4mm) at 37°C for 5 minutes prior to adding the other ingredients. Where specified, BSA (0.2%) was added with the enzyme. The extent of the digestion was assessed by running 0.5µg of digested DNA on an agarose gel and staining with ethidium bromide (50µl of 10mg/ml

ethidium bromide to 500ml buffer). The gel was stained for 30 minutes then observed on a UV transilluminator.

AGAROSE GEL ELECTROPHORESIS

DNA fragments were separated by vertical or horizontal agarose gel electrophoresis in 1 x TBE. The percentage of agarose added was varied to achieve the best separation of the required fragments. The gels were run at 25 to 35V overnight.

SOUTHERN BLOTTING

DNA was transferred to nylon membranes (Hybond, Amersham), after first depurinating for 2 x 15 minutes (in 0.25N HCl), then neutralising for 2 x 15 minutes (in 0.5N NaOH, 1M NaCl), and then equilibrating for 30 minutes (in 0.5M Tris-Cl, 3mM NaCl pH 7.4). It was blotted overnight by capillary action (in 1.5M NaCl, 0.15M NaCitrate pH 7.0). The DNA was attached to the nylon by UV cross-linking for 5 to 6 minutes on a short wave UV transilluminator. (The cross-linking time was worked out empirically). The filters were hybridized immediately or stored dry at room temperature.

LABELLING OLIGONUCLEOTIDES

The oligonucleotides were end labelled using $\gamma^{32}P$ dATP and T4 polynucleotide kinase (Amersham). 30ng of oligonucleotide were labelled in 25µl using 50µCi of $\gamma^{32}P$ dATP > 3000 Ci/mM (which was one molecule of $\gamma^{32}P$ dATP per molecule of oligonucleotide) and 5 units of T4 kinase. The

buffer was 10mM $MgCl_2$, 50mM Tris-Cl pH 7.6. The reaction incubated at 37°C for 10 minutes. EDTA was added to 10mM, the reaction was then diluted to 100µl with TE and extracted once with phenol/chloroform (1:1) and once with chloroform. Alternatively the probe was diluted to 500µl with TE and separated from unincorporated nucleotides using a NAP-5 column (Pharmacia). The incorporation was measured before and after adding the enzyme by spotting 1µl onto a square of 3mm paper, washing in 4 x 250ml cold 5% TCA, then in ethanol and ether. After drying at room temperature, scintillant (7.5g PPO, 750mg dimethyl POPOP in 2.5l toluene), was added and the cpm were measured using a scintillation counter. Routinely >80% of the label was incorporated.

MEASURING THE LENGTH OF OLIGONUCLEOTIDES

The labelled oligonucleotide (a 1/100 dilution of the labelling reaction) was run on a 25% acrylamide gel, containing 50% urea and 1 x TBE at 1000V for approximately 1 hour. The gel was fixed for 5 minutes in 10% methanol 10% acetic acid and autoradiographed for 5 minutes at room temperature. Other labelled oligonucleotides were used as markers.

DNA FRAGMENT PROBES

The group 1 and group 3 specific probes contained exon 1 and half of exon 2 up to the EcoRI site from the cDNA clones cLiv1 (group 1; Shahan *et al.*, 1987) and Mup 15 (group 3; Chave-cox, 1986). The Apolipoprotein AI probe was a 300bp partial cDNA clone from the 3' end of ApoAI including a stretch of 60 A residues (LVB6; Clissold and Bishop 1982). The PRL-R probe was a 719bp AvaII to XbaI fragment of the rabbit PRL-R

cDNA (Edery *et al.*, 1989) which was 77% homologous to the mouse PRL-R cDNA (Davis and Linzer, 1989).

LABELLING RESTRICTION FRAGMENTS

Restriction fragments were labelled by the method of Feinberg and Vogelstein (1984). The labelling buffer (OLB) contained a 2:5:3 mixture of A:B:C.

A: 625µl 2M Tris-HCl pH 8.0, 25µl 5M $MgCl_2$, 350µl H_2O, 18µl 2-mercaptoethanol, 5µl each of dATP, dTTP, dGTP (0.1M in 3mM Tris-HCl, 0.2mM EDTA pH 7.0). (Store at -20°C).

B: HEPES pH 6.6 (with NaOH) filter sterilized (Store at 4°C).

C: Hexadeoxyribonuceotides (Pharmacia) 900D units/ml in 3mM Tris HCl, 0.2mM EDTA pH 7.0.

The reaction was set up as follows: The DNA (30ng) and water to a final volume of 12µl were boiled for 3 minutes to denature the DNA, then chilled on ice. 6µl of OLB, 1.2µl of 10mg/ml BSA (enzyme grade), 4µl (40µCi) of α-^{32}P dCTP (>3000 Ci/mMol) and 1µl of klenow (1 unit) were added. The reaction was incubated overnight at 25°C.

MEASURING INCORPORATION INTO FRAGMENTS

Incorporation of α-^{32}P dCTP into DNA fragments was assessed by adding 1µl of the labelling reaction to 1ml 10% tetrasodium pyrophosphate, then adding 50µl 5mg/ml BSA and 150µl 100% TCA. DNA was precipitated on ice for 15 minutes then filtered onto a GFC filter disc. It was washed with 100ml 5% TCA. The radioactivity was measured by Cherenkov counts in a scintillation counter. $>10^8$ counts/µg were routinely obtained.

HYBRIDIZATIONS

These were done using the method of Church and Gilbert (1984). The hybridization buffer contained 0.25M $NaHPO_4$ pH 7.2, 7% SDS and 1mM EDTA. To reduce the background hybridization of oligonucleotides to rRNA 10µg/ml yeast tRNA were added to the hybridization buffer. Filters were pre-hybridized for 1 hour. Hybridizations were incubated overnight in a volume of up to 25mls per filter. The temperature of oligonucleotide hybridizations depended on the length and composition of the oligonucleotide. The temperatures which allowed discrimination between two sequences varying by one nucleotide were calculated and then determined empirically. The temperatures used were; oBS1 52°C RNA and DNA, oBL1 42°C RNA, 52°C DNA, oBL1RI 39°C RNA and DNA. DNA Fragment probes were hybridized at 68°C except for the PRL-R cDNA probe where the homology of the probe to the target was only 77% (60°C). Washes were carried out at the same temperatures as the hybridizations. Filters hybridized with oligonucleotides or the PRL-R probe, were washed as follows; two washes, 10 minutes in 500mls of 1mM EDTA, 0.25M $NaHPO_4$ pH 7.2 ,5% SDS; two washes, 20 minutes in 1l of 1mM EDTA, 0.25M $NaHPO_4$ pH 7.2, 1%SDS. Filters hybridized with DNA fragment probes were washed as follows; two washes, 10 minutes in 500mls 1mM EDTA, 20mM $NaHPO_4$ pH 7.2, 5% SDS, three washes, 20 minutes in 1l 1mM EDTA, 20mM $NaHPO_4$ pH 7.2 1% SDS. High stringency washes were used to distinguish group 1 from group 3. These were; one wash, 20 minutes in 1mM EDTA, 20mM $NaHPO_4$ pH 7.2, one wash, 20 minutes in 1mM EDTA, 6.5mM $NaHPO_4$ pH 7.2.

DNA FRAGMENT ISOLATION

Digested plasmids were electrophoresed overnight on agarose gels then

stained with 0.5 ug/ml Ethidium bromide in gel buffer for 15 minutes. In order to locate the DNA fragments of interest but minimize DNA damage, gels were viewed through a thin glass plate using a long wave UV transilluminator. A well the length of the DNA band and approximately 3mm wide was cut on the anode side and an L-shaped piece of dialysis membrane was inserted with the bottom of the L facing the cathode. The well was filled with buffer. Electrophoresis was continued using filter paper wicks for 60 minutes (gels up to 1%) or 90 minutes (gels more than 1%). The dialysis membrane and buffer were placed in approximately 2ml of 0.2M NaCl, 20mM Tris pH 7.5, 1mM EDTA. The DNA fragment was purified using an Elutip column (Schlierer and Schule) and precipitated with 4 volumes of ethanol for 30 minutes at -70°C or with 2 volumes of ethanol overnight at -20°C. The recovery of the fragment was assessed by comparison with dilutions of the original digest on an agarose gel.

LIGATION REACTIONS

Ligations were carried out in a volume of 20µl containing vector >0.5pmol/ml and insert >1pmol/ml. Ligation buffer was 66mM Tris pH 7.6, 6.6mM $MgCl_2$, 10mM DTT, 0.5mM ATP. For blunt end ligations polyethylene glycol (PEG) was added (5%). Ligations were carried out at 16°C overnight.

TRANSFORMATION OF BACTERIA

A single colony was inoculated into 10ml L-broth and grown overnight. This was used to inoculate a 25ml culture which was grown to mid-log phase (OD 650nm = 0.5). The culture was chilled and bacteria pelleted

by centrifugation at 10K for 8 minutes at 4°C. The bacteria were resuspended in half volume of ice cold 0.05M $CaCl_2$. The pellet was vortexed prior to adding $CaCl_2$ to aid resuspension. The bacteria were incubated on ice for 15 minutes then recentrifuged and resuspended in 0.1 volume of 0.05M $CaCl_2$. The competent cells were stored on ice for at least 45 minutes or up to one week in a refrigerator. 5 to 0.05ng of DNA was incubated on ice with 100µl competent for 15 minutes followed by a heat shock at 37°C for 2 minutes. 1ml of L-broth (37°C) was added at and the bacteria were incubated for 45 minutes at 37°C to allow expression of resistance genes. Bacteria were centrifuged for 10 seconds in a microcentrifuge, resuspended in 200µl L-broth and plated onto agar plates containing 100µg/ml antibiotic. For blue/white selection of β-galactosidase activity, 50µl of X-gal 20mg/ml in dimethylformamide and 20µl of IPTG 24mg/ml in H_2O were added per 400mls. Plates were inverted and incubated overnight.

PREPARATION OF PLASMID DNA

The alkaline lysis method from Sambrook *et al.*, (1989) was used to prepare mini-preps of plasmid DNA. Large scale preparation of plasmid DNA was done as follows: 25mls of an overnight culture of bacteria containing the plasmid was used to inoculate 250mls of L-broth containing antibiotic in a 2l flask which was shaken at 37°C for 2 hours. Chloramphenicol 150µg/ml was added and incubation was continued overnight. The next day the culture was chilled on ice then centrifuged at 10K, 4°C for 30 minutes. The bacterial pellet was resuspended in 20mls of 10mM Tris-HCl, 1mM EDTA pH 7.0 then centrifuged at 10K, 4°C for 3 minutes. The bacterial pellet was resuspended in 4mls of sucrose mix (25% sucrose, 50mM Tris HCl pH 8.1, 40mM EDTA pH 8.1) and 1.2ml of 10mg/ml lysozyme in sucrose mix were added. The bacteria were incubated

on ice for 15 minutes with occasional mixing. 1.2mls of 0.5M EDTA pH 8.1 were added and incubation continued on ice for 5 minutes. 10.8mls of triton mix (2ml 10% triton, 25ml 0.5M EDTA pH 8.1, 10ml 1M Tris pH 8.1 in 100mls) were added. The genomic DNA was removed by centrifugation at 20K, 4°C for 60 minutes. 17.1g of Caesium chloride and 1.8mls of ethidium bromide 10mg/ml were added to 18mls of supernatant. The plasmid DNA was banded by spinning in an A50 rotor at 35K for 3 days at 25°C. Bands containing plasmid DNA were viewed using a long wave UV light and removed with a syringe and needle. The ethidium bromide was extracted with isopropanol saturated with water and caesium chloride. The plasmid DNA was dialysed overnight in two changes of 10mM Tris-HCl and then purified over a sepharose 2B column. Column buffer contained 0.3M NaCl, 50mM Tris pH 7.4. Fractions containing the plasmid were located by absorption at 260nm. The plasmid DNA was then precipitated with 2 volumes of ethanol.

ISOLATION OF LIVER RNA

Total liver RNA was used for northern analysis.

This method was adapted from Chirgwin *et al.*, (1979).

Approximately 1g of tissue or 2 plates of tissue-culture cells were homogenised in 4ml of 4M guanidinium isothiocyanate containing: 25g guanidinium isothiocyanate, 1.25ml 1M sodium citrate pH 7.0, 0.25g sarkosyl, 0.165ml sigma antifoam A per 50ml. After filtration the pH was adjusted to 7.0 with a few µl of 8N NaOH. This solution was stored for up to one month at room temperature. The RNA was purified by centrifugation through a caesium cushion: 1.2ml of 5.7M CsCl, 25mM NaAc were placed in an SW50 tube and 4ml of homogenate were layered over the top. Balanced tubes were centrifuged for 12 hours at 36,000 rpm, 20°C. After centrifugation the band containing DNA was carefully removed from the interface and the supernatant was decanted. The pellet of RNA was

resuspended by pipetting in 1ml of 7.5M guanidine hydrochloride made up as follows: 35.81g guanidinium HCl made up to 50mls and then mixed with 1.25ml of 1M Na_2Citrate pH 7.0 and 1.25ml 0.2M DTT. The RNA was precipitated by adding 25µl of 1N Acetic acid and 0.5ml ethanol and incubating at -20°C overnight. RNA was pelleted by centrifugation in an eppendorf centrifuge at 8°C and resuspended in 1ml sterile ddH_2O. It was reprecipitated by adding 100µl of 2M NaAc pH 5.0 and 3.3ml Ethanol and incubating at -20°C overnight. RNA was stored in 70% ethanol at -20°C.

PURIFICATION OF MESSENGER RNA BY OLIGO dT CELLULOSE

Purified mRNA was used for *in vitro* translations.

This method was adapted from Berger and Kimmel (1989) p254-261.

0.1 to 1g of oligo dT cellulose (Sigma) were resuspended in 1 to 5ml 10mM Tris pH 7.4, 1mM EDTA. (1g binds 20 to 40 A_{260} units of pA^+ RNA in 0.5M NaCl at 0°C, pH 7.5). The cellulose was placed into a small column and was washed overnight with DEPC treated (0.01%) sterile ddH_2O. The RNA was resuspended in 10mM Tris pH 7.4, 1mM EDTA at 1mg/ml. An equal volume of 2x binding buffer was added then it was heated to 70°C for 5 minutes to denature the RNA. (Binding buffer contained 0.5M Lithium chloride, 0.5% SDS, 0.01M Tris pH 7.4, 1mM EDTA). The column was washed with 10mls binding buffer and then the RNA was applied three times. Following this it was washed with 15mls binding buffer and the pA^- RNA was collected in fractions of 1ml. The column was then washed with 10mls of wash buffer (0.01M Tris pH 7.4, 0.1M LiCl, 1mM EDTA) and the pA^- RNA was collected. Finally, the pA^+ RNA was eluted from the column with sterile DEPC treated TE and collected in 1ml fractions. The fractions containing RNA were identified by absorbance at 260nm and the RNA was precipitated by adding 0.1 volume of 2M NaAc pH 5.0 and 2 volumes of ethanol. The column was washed with 20ml 0.1N NaOH to

hydrolyse any remaining RNA prior to reuse. pA^+ RNA made up between 5 and 8% of total RNA. The pA^- and pA^+ RNAs were analyzed by Northern blotting and hybridization which showed that the majority of MUP message was in the pA^+ RNA fraction.

RNA GEL ELECTROPHORESIS

RNA was electrophoresed on a 1.8% agarose gel containing 6.4% formaldehyde and 0.01M phosphate pH 7.0. The running buffer contained 0.01M phosphate pH 7.0. The RNA was denatured in 5% formaldehyde, 24% formamide, 0.01M phosphate pH 7.0 at 60°C for 5 minutes then chilled on ice prior to loading. The gels were run at 30V overnight.

IN VITRO TRANSCRIPTION

The cDNAs were cloned into the vectors pTZ18R (Mead and Szczesna-Skorupa, 1986) (cLiv6 and cLiv1) or pT7-1 (Tabor and Richardson, 1985) (Mup15). T7 polymerase will start transcripts preferentially with a cap analogue, however, uncapped RNA can also be translated *in vitro*. The plasmids were linearized at the BamHI site downstream of the insert, extracted with phenol/chloroform (1:1) and precipitated using ethanol. The RNA was transcribed from 1µg of plasmid DNA in 50µl for 180 minutes at 37°C. The transcription buffer contained 40mM Tris pH 7.6, 15mM $MgCl_2$, 5mM DTT, 0.5mg/ml BSA, 1mM rNTPs and 1mM cap analogue $m^7G(5')pp-p(5')G$, 50 units of RNasin and 30 units of T7 RNA polymerase. 0.05µ Ci/µl ^{32}P rUTP were added to one tenth of the transcription reaction and 1µl samples were removed at 30 minute intervals. The incorporation was determined as described above for DNA fragment probes. Transcription was stopped by addition of 15mM EDTA and then phenol/chloroform

(1:1) extracted. RNA was precipitated with ethanol, resuspended in 100µl of 100mM NaAc pH 5.0, 5mM $MgCl_2$, 50 units of RNasin, 10 units of RNase free DNaseI and incubated at 37°C for 10 minutes. After digestion of DNA, EDTA was added to 5mM and the RNA was again phenol/chloroform extracted. The size of the transcripts were assessed by RNA gel electrophoresis (as above) using other *in vitro* transcribed RNAs as size markers. The amount of RNA synthesized was calculated from the incorporation of ^{32}P UTP and also by measuring the absorption at 260nm. Approximately 20µg of RNA was synthesized which was both homogeneous and full length.

URINE COLLECTION

Urine was collected by bladder massage. It was dialysed overnight against 10mM Tris pH 7.6, 50mM NaCl, then overnight against distilled water. The protein concentration was measured by the method of Lowry (1951) using BSA as a standard. 10µl of male urine or 50µl of female urine in 200µl were added to 1ml of Lowry C. Lowry C contained 25ml of Lowry A + 0.5ml Lowry B. Lowry A (2g Na_2CO_3, 0.4g NaOH, 20mg Na,K,Tartarate in 100ml); Lowry B (0.5g $CuSO_4.5H_2O$ in 100ml). After 15 minutes at room temperature, 0.1ml of 50% Folin and Ciocalteaus reagent were added. After exactly 20 minutes at room temperature, the OD was measured at 720nm. Urine was stored frozen at -20°C. To concentrate samples they were lyophilised for 1 to 2 hours in a vacuum drier and resuspended in a small volume of distilled water.

IN VITRO TRANSLATION

Translation reactions were done using a Pelham and Jackson (1976)

reticulocyte lysate and canine pancreatic microsomal membranes (Amersham). The reaction contained: Lysate 20µl, poly A+ RNA 2.5µg (5µl), membranes or membrane blank (20µl), ^{35}S-methionine 5µCi/µl (2.5µl) (>3000 Ci/mMol). 1µl samples were taken after 90 minutes using a 5µl capillary. The samples were hydrolysed in 1ml 1.0 M NaOH, bleached with 100µl 30% H_2O_2 and precipitated with TCA (10% final volume) for 30 minutes on ice. The precipitate was filtered onto GFC filters, washed with 100ml 5% TCA then dried in a vacuum oven. The radioactivity was measured using scintillant (as above) and counting on the ^{14}C cycle in a scintillation counter. The concentration of RNA used was determined empirically. The proteins synthesized were stored frozen at -20°C or -70°C.

ONE-DIMENSIONAL FLAT BED ISOELECTRIC FOCUSING PAGE.

Two thicknesses of IEF gel were used. The gels shown in Chapter 2 were 1.5mm thick. The gels shown in Chapters 3 and 4 were 0.75mm thick. The method used was from "Polyacrylamide Gel Electrophoresis, Laboratory Techniques" (pharmacia) page 32. Gels 1.5mm and 0.75mm thick had volumes of 15ml and 7.5ml respectively. The stock acrylamide solution (50ml) contained 4.85g acrylamide, 0.15g bisacrylamide and was deionized for 1 hour at room temperature with 0.5g MB3 resin per 50ml. It was filtered and stored for up to one week at 8°C. 30ml of gel solution contained 4ml glycerol, 9.1ml double distilled water, 15ml stock acrylamide and pharmalytes. Different combinations of pharmalytes were used to give the best separation. Either 0.27ml pharmalyte 4 to 6.5 and 1.63ml pharmalyte 4.2 to 4.9, or 1.8ml pharmalyte 4.2 to 4.9 and 0.2ml pharmalyte 3.5 to 5, were used. The gel solution was degassed then 200µl of ammonium persulphate (22.8mg/ml) were added. The gels were poured using a dual slab gel electrophoresis apparatus (BioRad)

and could be stored for up to two months at 8°C or used immediately. Gels were transferred to plastic sheets (Pharmacia PAGE-plate kit) and placed onto the Pharmacia flat bed gel electrophoresis apparatus which was pre-cooled to the running temperature (10°C) using a refrigeration unit and pump. The electrode wicks were made from 1mm thick BioRad gel drying paper cut into strips 0.5cm x 12cm. Two strips were used for each electrode. The electrode wicks were soaked in electrode buffer at 8°C. The electrode buffers were according to Pharmacia, for the longest pH gradient in the gel; pH 4 to 6.4 or pH 3 to 5.5.

pH 3 to 5.5; anode 1M H_3PO_4, cathode 1M glycine.

pH 4 to 6.5; anode 0.01M DL-Glutamic acid, cathode 0.01M histidine.

The electrode strips were blotted on 3mm paper, then placed on the gel surface at the anode or cathode respectively. The gel was pre-run for 1 hour at 10mA, 1400V. The samples (up to 20µl) were loaded onto Whatman no1 filter paper squares 1cm x 0.5cm which were placed onto the surface of the gel 1cm from the cathode electrode strip. The gel was run for 2 to 3 hours at 10mA, 1400V, fixed for 30 minutes in 57.5g TCA, 17.25g sulphosalaicylic acid, 500ml water, rinsed in destain (500ml ethanol, 160ml acetic acid, to 2l with water) and stained in 400ml stain heated to 60°C (100ml of 4.6g/l coomassie blue filtered through Whatman no1 paper, 32ml acetic acid, water to 400ml). Gels were destained at 60°C, then dried on a BioRad gel drier at 80°C.

ROD GEL ISOELECTRIC FOCUSING

In The Presence Of Urea

This was done as described in Hames and Rickwood (1981) pp 201-203 with the following modifications;

Instead of 0.4ml 40% ampholines pH 5 to 7 and 1ml 40% ampholines pH 3.5

to 10 the following were used; gels contained 490µl pharmalyte 4.2 to 4.9, 80µl pharmalyte 4 to 6.5. Sample buffer (per 1ml) contained 32µl pharmalyte 4 to 6.5, 196µl pharmalyte 4.2 to 4.9.

The rod gels were cast in glass tubes 13cm long x 3mm internal diameter. 1ml of gel solution was used per gel. The following electrode solutions were used; cathode, 0.01M Histidine, anode, 0.01M glutamic acid. Sample overlay was not used with these electrode solutions. Gels were run at 25°C in a warm room. The gels were pre-focused at 200V for 15 minutes, 300V for 30 minutes and 400V for 1 hour. They were run at 400V for 14 to 18 hours then 800V for 1 hour.

In the absence of urea

10mls of gel solution contained 1.33ml 28.38% acrylamide, 1.62% bisacrylamide, 0.5ml glycerol, 7.583ml ddH_2O, 80µl pharmalyte 4 to 6.5, 490µl pharmalyte 4.2 to 4.9, 10µl 10% ammonium persulphate, 7µl TEMED. Rods and electrode solutions were as above. The samples contained: 5% glycerol, 0.8% pharmalyte 4 to 6.5, 4.9% pharmalyte 4.2 to 4.9. Gels were run as above.

2D GEL ELECTROPHORESIS

Prior to second dimension, rod gels, as above, were equilibrated in 10mls of equilibration buffer. This contained 10mls glycerol, 5mls β-mercaptoethanol, 11.5mls 20% SDS, 5mls 1.25M Tris pH 6.75, 2.5ml 0.2M EDTA, 1ml 0.5M EGTA, 1ml 37mg/ml PMSF in isopropanol per 100ml.

Equilibration was carried out for 1 hour at room temperature on a shaking table. Gels were overlayed on the second dimension gel or stored at -70°C in equilibration buffer.

SDS DIMENSION

Stock 30% acrylamide contained 29.2g acrylamide, 0.8g bis-acrylamide per 100ml. Laemmli-type discontinuous gel buffers were used. The resolving gel was 15% acrylamide, 0.375M Tris-HCl pH 8.8, 0.1% SDS. It was 1.5mm thick and 12cm long. The stacking gel was 4.8% acrylamide, 0.125M Tris pH 6.75, 0.1% SDS. Gel buffer was Tris glycine, this was; 1D gels: 0.05M Tris, 0.384M glycine 0.1% SDS, pH 8.3; 2D gels: 0.025M Tris, 0.192M glycine, 0.1% SDS pH 8.3.

The rod gel was attached to the slab gel with 1% agarose (IEF grade) made up with equilibrium buffer. Running dye (1ml bromophenol blue 1% in ethanol in 9ml equilibration buffer) was laid along the top of the gel. The second dimension slab gels were run at 70V overnight. One dimensional slab gels were run for 12 hours at 60V or overnight at 45V. Gels were stained as for slab IEF gels. After staining gels were soaked in 500ml destain containing 7% glycerol. Radioactive gels were soaked in amplify (Amersham) for 30 minutes. Gels were dried for 2.5 to 3 hours at 50°C on the gradient cycle of a BioRad gel drier.

MOUSE STRAINS

C57BL/6 mice were obtained from Banton and Kingman. Balb/c mice were purchased from MRC laboratory animals centre, Carshalton, Surrey on 22.09.71 and were maintained in the mouse house at Edinburgh University Genetics Department. *Lit/lit* mice were on a C57BL/6 background and were maintained in the Mouse House at Edinburgh University Genetics Department.

HORMONE TREATMENT

Male *lit/lit* mice were treated with ovine or bovine GH (gift of U.S. National Pituitary Hormone Progamme) which was made up as indicated by the supplier. Mice were injected itraperitoneally with 100µg per 20g body weight every 12 hours for 5 days or infused with a sub-cutaneous minipump (Alzet model 1003D) with 5µg GH (1µl) per hour per 20g body weight for three days.

BACTERIAL STRAINS

E.Coli strain HB101 (supE44, ara-14, proA2, hsdS20, recA13, r_B^- m_B^-, lacY1, galK2, rpsL20, xyl-5, mtl-1) was used to grow plasmids.

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