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Regulation Of Mitogen-regulated Protein/ proliferin Gene Expression In Cultured Mouse Cells

Alison Margaret Connor

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REGULATION OF MITOGEN-REGULATED PROTEIN/PROLIFERIN GENE EXPRESSION IN CULTURED MOUSE CELLS

BY

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Abstract

Mitogen-regulated protein/proliferin (MRP/PLF), a member of the prolactin-growth hormone family that occurs in the mouse, is expressed in a number of immortal cell lines including 3T3 and BNL. Neither MRP/PLF protein nor mRNA is detected in the mouse embryo fibroblasts (MEFs) from which the 3T3s are derived. One aim of this work was to identify the level at which this regulation of MRP/PLF expression is occurring. Differences were not observed in the MRP/PLF gene copy number, DNase I hypersensitive sites or methylation patterns of immortal cell lines compared to MEFs. RNA transcriptional mapping did not detect MRP/PLF hybridizing transcripts in either the nucleus or cytoplasm of the MEFs. These results, coupled with previous results that MRP/PLF is transcribed to an equal extent in both MEF and 3T3 cells, led to the conclusion that the MRP/PLF transcripts are highly unstable in MEF cells.

MRP/PLF expression in 3T3 and BNL cells is induced by a variety of growth factors but little is known about how the growth factors bring about changes in MRP/PLF expression.

An MRP/PLF gene was isolated from a mouse genomic library. Sequencing and Southern blot analysis allowed the determination of the intron/exon structure. S1 mapping identified the transcriptional start site and 1.1-Kbp of the promoter region was sequenced. Potentially important

regions were identified by comparison with reported regulatory sequences. Transient expression assays revealed that the promoter is weak but functional, and likely contains a negative regulatory element in a defined 65-bp region. Northern blotting showed that MRP/PLF cytoplasmic RNA levels are negatively regulated by dexamethasone and positively regulated by TGF-α, estrogen and to a small extent, vitamin D3. Suitable clones were generated so that transient expression assays can be used to further define the potentially important regulatory regions.

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Table of Contents

List of Tables

List of Figures

LIST OF ABBREVIATIONS

A	alanine
A	adenine
ADP	adenosine 5'-diphosphate
Ala	alanine
Ap	ampicillin
Arg	arginine
Asn	asparagine
ATCC	American Type Culture Collection
Asp	aspartic acid
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
cAMP	adenosine 3',5'-cyclic phosphoric acid
C	cysteine
CAT	chloramphenicol acetyl transferase
cpm	counts per minute
Cys	cysteine
D	aspartic acid
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
ddATP	dideoxyadenosine 5'-triphosphate
ddCTP	dideoxycytidine 5'-triphosphate
ddGTP	dideoxyguanosine 5'-triphosphate

ddTTP	dideoxythymidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
E	glutamic acid
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetra-acetic acid
F	phenylalanine
FGF	fibroblast growth factor
G	guanine
G	glycine
G0	quiescent state of cell cycle
G1	gap in cell cycle between mitosis and DNA synthesis
G2	gap in cell cycle between DNA synthesis and next mitosis
GH	growth hormone
Gln	glutamine
Glu	glutamic acid
Gly	glycine
H	histidine

HBS	Hepes buffered saline
Hepes	(N-2-Hydroxyethylpiperezine-N'-2-Ethane Sulfonic Acid)
His	histidine
I	isoleucine
IGF-I	insulin like growth factor I/Somatomedin C
IGF-II	insulin-like growth factor II
Ile	isoleucine
IPTG	isopropyl β-d-thio-galactopyranoside
K	lysine
Kbp	Kilobase pair
Klenow	E. coli DNA polymerase II-Klenow fragment
L	leucine
L.broth	Luria broth
Leu	leucine
Lys	lysine
M	methionine
M	mitosis
MEFs	mouse embryo fibroblasts
Met	methionine
moi	multiplicity of infection
MOPS	(3-[N-Morpholno]propanesulfonic acid)
Mr.	molecular weight
MRP	mitogen-regulated protein
N	asparagine
NEN	New England Nuclear

mRNA	messenger ribonucleic acid
P	proline
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PEG	polyethylene glycol
pfu	plaque forming unit
Phe	phenylalanine
Pipes	(Piperazine-N,N'-bis[2-ethane-sulfonic acid])
PKC	protein kinase C
PL	placental lactogen
PLF	proliferin
polI	E. coli DNA polymerase I
PRL	prolactin
Pro	proline
Q	glutamine
R	arginine
RNA	ribonucleic acid
rPLP-A	rat prolactin-like protein A
S	onset of DNA synthesis
S	serine
SDS	sodium dodecyl sulphate
Sequenase	modified T7 DNA polymerase
Ser	serine
ss	single-stranded
SSC	standard saline citrate (NaCl 0.15 M,

	NaCitrate 15mM)
T	threonine
T	thymine
TBS	Tris-buffered saline
TE	10 mM TrisHCl (pH 8), 1mM EDTA
Thr	threonine
TLC	thin layer chromatography
TPA	12-O-tetradecanoylphorbol-13-acetate
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet light
V	valine
Val	valine
vol	volume
W	tryptophan
Y	tyrosine

CHAPTER 1

INTRODUCTION

1.01 Growth Factors

Polypeptide growth factors such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and the insulin-like growth factors (IGF) affect the growth and differentiation of animal cells in both the organism and in culture. They have been implicated in a wide variety of physiological and pathological processes including selective cell survival, hematopoiesis, tissue repair or wound healing, immune responses, atherosclerosis, neoplasia, embryogenesis, and growth and development (Rozengurt, 1986). More recently, evidence suggests that they may also control differentiation and morphogenesis during mammalian development (Mercola and Stiles, 1988). They are found in platelets and tissues both of adult and fetal origin and are released by many cells in culture. Some factors, such as those of the hematopoietic system, stimulate only a few cell types while others such as EGF and IGF are effective on numerous cell types.

The high-affinity trans-membrane receptors for many of the growth factors are tyrosine-specific protein kinases that are activated upon binding of the growth factor to its receptor to phosphorylate not only themselves but also

intracellular substrates (Buhrow et al., 1982, Heldin et al., 1983, Roth and Cassell, 1983, Heldin and Westermark, 1984). These receptors subsequently undergo redistribution in the membrane and endocytosis so that the ligand-receptor complexes can be transported to the Golgi apparatus where they are segregated into the lysosomal pathway (Rozengurt, 1983).

The major receptor for tumour promoters of the phorbol ester family is protein kinase C (PKC), a calcium-sensitive, phospholipid-dependent protein kinase which acts on serine and threonine residues. PKC is activated in vivo by diacylglycerol, which is generated by phospholipase C action on membrane phospholipids (Nishizuka, 1984). A number of other mitogens, including serum, PDGF, and FGF can also rapidly activate PKC.

Biochemical events that occur in response to growth factor-receptor binding are the formation of phosphoinositide metabolites, an increase in the influx of sodium, which is partly coupled to the exit of hydrogen ions thereby resulting in a rise in the cytoplasmic pH, an elevation in potassium ion uptake, and a rapid two-to-threefold transient increase in cytosolic calcium levels (Rozengurt, 1986). The activation of PKC also either directly or indirectly results in increased ion fluxes across the plasma membrane. One or more of these events leads to the generation of an intracellular signal which is propagated to the nucleus where a set of specific growth-

related genes is activated followed by the temporal expression of other genes and subsequent DNA synthesis and cell division (Goustin et al., 1986). All of this occurs within ten to fifteen hours of growth factor addition.

1.02 The Cell Cycle

Many workers have described what is known as the cell cycle in order to characterize the events that occur between one cell division and the next. Since DNA synthesis can be measured quite readily by the use of autoradiography to follow the incorporation of tritiated thymidine into nucleic acid, the cell cycle is normally described in terms of DNA synthesis. Typical mammalian cells that are continuously proliferating or cycling usually have an interval, known as G1, lasting 5 to 10 h between the completion of mitosis (M) and the onset of DNA synthesis (S). There is no DNA synthesis during G1 but there is synthesis of new mRNAs and proteins that are required for progression through G1 (Baserga, 1985). Another gap, known as G2, occurs between the S phase and the beginning of the next mitosis. Cells can be induced to withdraw from the cell cycle by serum starvation or density inhibition and enter a state known as G0 or quiescence. The cells of many tissues and organs exist in a G0-like state where they are not proliferating but they are viable and metabolically active. Cells in G0 reduce the synthesis of enzymes required for cell duplication.

If quiescent cells are given a stimulus such as the addition of growth factors, hormones, or antigens they can re-enter the cell cycle at the G1 point (Pardee et al., 1978). Cultured murine 3T3 cells have been particularly useful in elucidating the cellular and molecular changes evoked in response to growth factors.

Studies carried out using combinations of growth factors have revealed that there are defined patterns of synergistic interactions between them. PDGF for example, in the absence of prior protein synthesis, can induce the expression of a set of genes within one hour of stimulation of resting mouse 3T3 cells with purified growth factor (Cochran et al., 1983). It is believed that some of these so called "competence genes", which include c-fos, c-myc, actin, KC, JE and a number of other genes whose function is presently unknown, induce a state in the cell that then allows it to respond to other growth factors known as "progression factors" and so proceed toward DNA synthesis and cell division.

It has been estimated that 3% of the RNA species in logarithmically growing mouse fibroblasts are absent in non growing cells (Williams and Penman, 1975). Differential screening of cDNA libraries has revealed that only about 0.3-1.0% of the genes screened are inducible by growth factors (Ferrari and Baserga, 1987). In other words, 50 to 100 genes are strongly dependent on growth factors (Linzer and Nathans, 1983). The small percentage of genes and gene

products which respond to growth factors are probably ultimately involved in regulating cell growth. If one could understand how the various growth factors and responsive genes interact in normal cells, it should enable one to understand the abnormal growth of cancer cells.

Since it is thought that the control of cellular proliferation resides in G1 (Pardee et al., 1978, Baserga, 1985), there is much interest in identifying genes which are expressed specifically during G1.

1.03 Growth Factor Regulated Genes

Over the past several years, a number of laboratories have set out to identify those genes or gene products that are regulated by growth factors. Several approaches have been used. The c-fos and c-myc genes, for example, were suspected of encoding regulatory proteins and therefore changes in the expression of these genes after serum stimulation was looked for (Kelly et al., 1983, Greenberg and Ziff, 1984). In other studies, the profile of new proteins, which were present after the cells were stimulated with growth factors, was observed by labelling the proteins and separating them by polyacrylamide gel electrophoresis (Nilsen-Hamilton et al., 1980, Thomas et al., 1981, Pledger et al., 1981). Still others have employed differential screening of libraries with probes made from RNA isolated from resting or stimulated cells to identify genes whose expression is altered after stimulation of the cells

(Cochran et al., 1983, Linzer and Nathans, 1983, Edwards and Denhardt, 1985). Some of the growth-regulated genes identified by the various methods are listed in Table 1. It can be seen that in those cases where a function is known, the growth-regulated genes encode proteins that are involved with the general metabolism and structure of the cell or are secreted. The remainder of this introduction will be concerned with one of the growth-regulated genes whose encoded protein is secreted, namely mitogen-regulated protein/proliferin (MRP/PLF).

1.04 Mitogen-Regulated Protein/Proliferin

Mitogen-regulated protein (MRP) was identified in 1980 as a family of glycoproteins which are secreted into the culture medium by Swiss 3T3 cells approximately 20 to 24 h after the addition of mitogens such as serum, fibroblast growth factor and epidermal growth factor and are detected by [^{35}S]methionine labelling (Nilsen-Hamilton et al., 1980). If tunicamycin, a specific inhibitor of N-linked glycosylation, is present, a 22,000 dalton protein representing the non-glycosylated form of MRP is observed. This is in contrast to the heterogeneously glycosylated forms of MRP normally seen having molecular weights of between 30,000 and 38,000 daltons. The release of MRP is obliterated by the transcriptional inhibitor actinomycin D suggesting that RNA synthesis is required prior to their release and/or synthesis. Hydroxyurea and butyrate, agents

Table 1
Growth-regulated genes[1]

Involved in DNA Synthesis	Involved in Cellular Structure and Metabolism	Receptors for Growth Factors	Secreted Proteins	Growth Regulated Oncogenes
	glyceraldehyde phosphate dehydrogenase	interleukin-2 receptor	mitogen-regulated protein/proliferin (MRP/PLF)	c-ras
thymidine kinase	enolase		major excreted protein (MEP)/cathepsin L	c-myc
thymidylate synthetase	triosephosphate isomerase		fibronectin[2]	c-fos
dihydrofolate reductase	ADP/ATP carrier		2AR-mouse osteopontin[3]	c-fgr
ornithine decarboxylase	calmodulin		tissue inhibitor of metalloproteinases (TIMP)[4]	p53
	β-actin		plasminogen activator	c-myb
	vimentin			
	calcyclin			
	transferrin receptor			

[1] From Denhardt et al., 1986 and Ferrari and Baserga, 1987, except:

[2] Blatti et al., 1988; [3] Craig et al., 1988; [4] Edwards et al., 1986.

which inhibit DNA synthesis, also prevent MRP release. Agents such as monensin, nigericin, ammonium chloride, methylamine and chloroquine, which increase intralysosomal pH thereby inhibiting lysosomal protein degradation, increase the amount of extracellular MRP (Nilsen-Hamilton et al., 1981). MRP levels thus appear to be regulated at a pre-translational level by growth factors and at a post-translational level by degradation in the lysosomes (Nilsen-Hamilton et al., 1981).

1.05 The Relation of Mitogen-Regulated Protein and Proliferin

A partial cDNA clone, isolated from a lambda gt11 expression library screened with an antiserum raised against MRP, was found to have a sequence identical to that of a proliferin (PLF) cDNA clone (Parfett et al., 1985). Further evidence that MRP and PLF are the same molecule was provided by the fact that an antiserum raised against MRP was specifically able to immunoprecipitate PLF secreted by CV-1 cells transfected with an expression vector encoding PLF (Nilsen-Hamilton et al., 1987).

The identification of proliferin began in 1983. An approximately 1 kb mRNA, that is barely detectable in resting cells but increases some 20 fold after serum stimulation reaching a maximum level at the G1/S boundary, was isolated by differential library screening (Linzer and Nathans, 1983). The protein encoded by this mRNA was later

named proliferin because of its perceived association with proliferation and sequence homology to prolactin (Linzer et al., 1984). Since description of the MRP protein preceded description of the PLF protein, in this thesis, Nilsen-Hamilton's suggestion will be followed and the protein will be referred to as MRP/PLF (Nilsen-Hamilton et al., 1987).

The maximum level of MRP/PLF mRNA is not attained until approximately 12 hours after serum stimulation, therefore its expression is not one of the early events in cells stimulated with mitogens and it is not considered to be one of the "competence genes". The induction of its expression requires prior protein synthesis. When quiescent cells were stimulated with serum in the presence of cycloheximide, a drug which inhibits translation, MRP/PLF mRNA was not detected. A time course revealed that protein synthesis was required for the first 4 to 6 hours of serum stimulation for MRP/PLF expression (Linzer and Wilder, 1987).

There are several pieces of evidence which suggest that the induction by growth factors of MRP/PLF mRNA and cellular proliferative responses are separable. Firstly, MRP/PLF mRNA is not detected in a number of actively growing cell lines (see 1.08). Secondly, serum stimulation of mouse embryo fibroblasts (MEFs) resulted in DNA synthesis and cell division but MRP mRNA was not detectable in the cytoplasm (Parfett et al., 1985). Thirdly, the addition of transforming growth factor-β (TGF-β) to

growing Swiss 3T3 cells had an inhibitory effect on the incorporation of [^{35}S]methionine into secreted MRP/PLF. This however, was not accompanied by an inhibition of DNA synthesis as followed by [^{3}H]thymidine incorporation (Chiang and Nilsen-Hamilton, 1986). Like c-myc, the levels of MRP/PLF mRNA and protein do not vary greatly during the cell cycle of normally cycling cells (Denhardt et al., 1986).

Although the function of MRP/PLF is unknown at present, the observations presented in sections 1.06, 1.07 and 1.08 suggest that it may be a placental hormone involved in the growth and differentiation of fetal or maternal tissue and an autocrine growth factor for cells in culture.

1.06 MRP/PLF and the Prolactin-Growth Hormone Family

The amino acid sequence of MRP/PLF deduced from its cDNA sequence reveals that MRP/PLF has 46% of its amino acids in common with bovine prolactin if closely related amino acids are included (Linzer and Nathans, 1984). In addition, MRP/PLF and prolactin have nearly identical locations of six cysteines and two tryptophans. Thus it has been proposed that MRP/PLF is a member of the prolactin-growth hormone family, which also includes the placental lactogens/chorionic somatomammotropins, proliferin-related protein (Linzer and Nathans, 1985) and a number of other prolactin-like proteins that have not been well characterized yet.

The prolactin-growth hormone family encompasses a group of polypeptide hormones related by structure, function, and immunochemistry. All of these hormones have a similar size (190 to 199 amino acid residues), are globular proteins and have a small loop of amino acids at the carboxy terminus. Each hormone has two homologous disulfide bonds. It is believed that these hormones arose by duplication of an ancestral hormone gene.

Growth Hormone: Growth hormone (GH) is produced in the anterior pituitary of vertebrates. It is one of the principal regulators of balanced post natal growth in vivo being involved in the development of bone from cartilage. It is also required for the maintenance of nitrogen, mineral, lipid, and carbohydrate metabolism. It initiates the utilization of fatty acids for energy and represses glycogenolysis. Growth-hormone-releasing hormone stimulates GH release while GH-inhibiting hormone inhibits GH release (Eckert and Randall, 1978). A family of closely related peptide growth factors called somatomedins or insulin-like growth factors are produced in response to circulating growth hormone.

Prolactin: Prolactin (PRL) is produced in the anterior pituitary gland by a specialized type of cell termed the lactotroph or mammotroph. In the early 1930's prolactin was reported to stimulate "crop milk" production in pigeons (Riddle and Braucher, 1931), an effect which became the basis of the bioassay of this hormone. Although PRL is

well known in controlling the growth and differentiation of the mammary gland and the prostate gland, many other physiological effects produced by PRL have been found both in males and females. These include osmoregulation in teleost fish, regulation of metamorphosis in amphibians, regulation of parental behaviour in teleosts, birds and mammals and promotion of growth in teleosts, amphibians, reptiles, birds and mammals. All of these actions require complex interactions of PRL and many other hormonally regulated events.

Prolactin receptors have been found in a wide variety of tissues and species. It is thought that the lactogenic hormones initially interact with their target cells by binding to "receptors" located on the outer surface of the plasma membrane. They are then rapidly taken up into target cells and bound to specific intracellular organelles, perhaps for degradation (Rillema, 1987). Various substances have been proposed to act as second messengers of PRL action including the monophosphate nucleotides, polyamines, prostaglandins and calcium/calmodulin. Recent evidence suggests that PKC is the second messenger for PRL action, since PRL-stimulated enzyme induction can be mimicked by TPA and inhibitors of PKC prevent PRL-stimulated mitogenesis in the Nb2 rat node lymphoma line (Buckley et al., 1988). Addition of PRL to rat liver results in the induction of ornithine decarboxylase (Richards, 1975) and plasminogen activator (Buckley et al.,

1984) which, as shown in Table 1, are both biochemical markers for cells entering the G1 phase of the cell cycle upon induction by growth promoting stimuli. Prolactin therefore, is able to stimulate cellular proliferation in non-reproductive mammalian organ systems and may in certain cases, function as a tumour promoter. Prolactin has been found to play a key role in mammary and prostatic tumourgenesis in rodents (Rillema, 1987).

Placental lactogen: Placental lactogen (PL) is produced in the placenta by trophoblast giant cells (Soares et al., 1985). In humans, there appears to be only one such protein which is closely related to GH. In rodents, there are at least two of these proteins which are antigenically dissimilar. PL-I is secreted during mid-gestation and has a molecular weight of 40,000 to 50,000. PL-II has a molecular weight of 20,000 to 23,000 and is secreted during the second half of gestation. Both of these hormones are potent lactogens and are more closely related to PRL than to GH (Duckworth et al., 1986a).

Since many of the functions of the pituitary hormones GH and PRL are required during pregnancy, it is possible that the placental lactogens might assist or replace the aforementioned hormones in events such as growth of the fetus and placenta, development of the mammary gland and maternal metabolism.

Other members of the growth hormone-prolactin family: Two PRL-like glycoprotein hormone complexes have been isolated

from the midpregnant mouse (Colosi et al., 1987). In addition, multiple low molecular weight PRL-like proteins have been found to be synthesized and secreted by rat trophoblast giant cell explants (Soares et al., 1988). One of these has the same molecular weight, 23,000, as a PRL-like hormone produced by the decidual tissue of the rat (Jayatilak et al., 1985). Both of these proteins bind to PRL receptors as shown by radio-receptor assays. The authors, however, fail to comment on whether these two proteins are homologous.

Recombinant DNA technology has allowed the identification of several other members of this family which have not been identified by classical receptor assays. Two members were found in the form of cDNA clones in a library made of placental RNA obtained from late gestation BALB/c mice. The corresponding mRNAs are predicted to encode proteins known as MRP/PLF and proliferin-related protein (PRP), both of which have substantial homology to the prolactin-growth hormone family (Linzer and Nathans, 1985). If it is assumed that the frequency of clones appearing in a library reflects the in vivo situation then MRP/PLF and PRP mRNAs are abundant placental RNAs, each representing 0.2 to 0.5% of the total poly[A^+] mRNA in mouse placenta.

A cDNA clone encoding a protein expressed in late-term rat placenta was isolated and termed rat prolactin-like protein A. In vitro translation of mRNA hybridizing to this clone resulted in the production of a protein of

25,000 daltons (Duckworth et al., 1986b). Multiple mRNAs encoding PRL-like proteins have also been found in the bovine placenta (Schuler and Hurley, 1987). Although the proteins have not been identified, it is predicted that one of them would consist of about 200 amino acids which is similar in size to the other members of this family. Perhaps the placental expression of multiple prolactin-like proteins is a common feature of many mammalian species with the exception of the primates which express a unique placental lactogen.

1.07 MRP/PLF Expression in the Mouse Placenta

In the mouse, MRP/PLF mRNA is present in the fetal component of the mid-gestation placenta which is comprised of the labyrinth, cytotrophoblast and giant cells. It is not present in the other tissues looked at which include the liver, kidney, ovary, pituitary gland, fetal brain and whole fetus. The MRP/PLF mRNA increases abruptly from day 8 to day 10 of pregnancy then decreases slowly until day 18 (Linzer et al., 1985). On day 8, the neural folds in the ectoderm are forming in response to inductive signals from the underlying mesoderm. There is also formation of somites and neuromeres. On day 9, there is acceleration of organogenesis and the contour of the embryo changes rapidly (Dony and Gruss, 1987). The MRP/PLF protein appears on day 9 and then rapidly declines between day 13 and 14 of development. This rapid decline in the amount of protein

present points to some form of post-translational control and Nilsen-Hamilton et al., (1988), have proposed that this is brought about by degradation in the lysosomes. Immunostaining of MRP/PLF protein and in situ hybridization to MRP/PLF mRNA has localized the site of synthesis and storage of MRP/PLF to the trophoblastic giant cells of the mouse placenta (Lee et al., 1988). These cells are also known to produce murine placental lactogen II and steroids. MRP/PLF has been found in the serum and amniotic fluid of pregnant mice using the radioimmunoassay technique (Lee et al., 1988). The level of MRP/PLF in the serum could be directly correlated with the number of placentas in a mouse suggesting that the placenta is the source of circulating MRP/PLF.

The placenta: The placenta is formed so that physiological exchanges may take place between fetal and maternal tissue. Since placentas differ among the species, the discussion here will be limited to the rodents.

The placental membrane consists of six layers. The trophoblast provides the basic fetal component of the placenta. It proliferates and "grows" into the endometrium by eroding the implantation site by lytic action. The trophoblast is also known to produce enzymes and hormones of vital importance to the maintenance of pregnancy and for fetal growth and development including murine placental lactogen II and MRP/PLF as previously mentioned. A third type of cell is the mesoderm which lies internal to the

cytotrophoblast. It forms the inner layer of the wall of the chorion. Wandering trophoblastic giant cells which may have 1 to 3 nuclei are present in various parts of the uterine wall. They appear to be derived directly from the cytotrophoblast and are next to maternal circulation (Ramsey, 1982). Table 2 lists some of the proteins and steroids expressed in the placenta. Several members of the growth hormone-prolactin family are present as well as some of the growth factors which have been shown to induce them. Also present are a number of the proto-oncogene products.

1.08 MRP/PLF Expression in Cultured Cell Lines

With the exception of GH and PRL synthesis by some pituitary tumour derived cell lines, MRP/PLF is the only known member of this family which has been found to be expressed by cultured cell lines. MRP/PLF is detected in a variety of actively growing established mouse cell lines including Swiss 3T3, BALB/c 3T3, NIH 3T3, 3T6, BNL, Erlich ascites cells, C3H, 10T½ and Krebs ascites carcinoma cells. It is not, however, detected in the JB6 epidermal line, B16F1 or 3T12 cells indicating that it is not essential for the establishment of a cell line. A curious observation is that MRP/PLF is not detected in MEFs, yet depending on the way they are passaged, it is (3T3 and 3T6), or is not (3T12) present in the cell lines derived from them (Parfett et al., 1985, Denhardt et al., 1987). This is discussed in greater detail in chapter 5. It has been suggested that in

Table 2

Proteins and Hormones Expressed in the Mouse Placenta

		Time of maximum expression
Hormones	estrogens	all stages
	insulin	all stages
	progesterone	all stages
	cortisol	all stages
Cellular proto-oncogene products	c-myc	early gestation
	c-fos	all stages
	c-sis	early gestation
	c-fms	day 14 and 15
	c-ras^{Ki}	days 12-18
	c-ras^{Ha}	all stages
Growth factors	TGF-α	day 7-9
	PGDF	early gestation
	TGF-β	all stages
	IGF-II	mid gestation
	CSF-2	----
	heparin binding growth factor (FGF)	day 7-9
Others	MRP/PLF	day 9-10
	Proliferin-related protein	day 12
	Placental lactogen I	mid gestation
	Placental lactogen II	late gestation

from Goustin et al, 1986; Muller et al, 1983; and Schindler, 1982.

some situations MRP/PLF may be acting as an autocrine growth factor (Linzer et al., 1985, Edwards et al., 1987). Autocrine growth factors: Most hormones such as insulin and adrenocorticotropic hormone are carried in the blood stream and act in an endocrine manner whereby they exert their actions at locations distant to their site(s) of origin (Darnell et al., 1986). Growth factors on the other hand, do not usually get carried in the blood stream but rather diffuse short-range to their target through intercellular spaces and act in a paracrine (on other near-by cells) or an autocrine (on cells that produced the factor) manner (Goustin et al., 1986).

Tissue culture systems have been used in the study of paracrine/autocrine regulation of growth since the cells can be grown in serum-free medium which they "condition" by the release of growth factors. The growth factor released by the cells can then be purified from the medium. Examples of growth factors which are capable of eliciting a paracrine or an autocrine response include the sarcoma growth factors later shown to belong to the transforming growth factors. They were identified in the conditioned medium of murine sarcoma virus transformed 3T3 cells as being able to stimulate growth of normal rat kidney cells (DeLarco and Todaro, 1978). PDGF-like growth factors were shown to be released into the medium by explants of human first trimester placenta. In addition cultured trophoblasts were found to express high affinity PDGF receptors

(Goustin et al., 1985). Somatomedin C/insulin-like growth factor-I, was found to be secreted into the medium by explant cultures from a variety of mouse tissues. Cultured human and rat fibroblasts also synthesize this factor (D'Ercole et al., 1984). Basic fibroblast growth factor is made by a number of cells in vivo as well as a number of types of cultured cells. It was shown that the release of bFGF by bovine aortic endothelial cells regulates their movement and basal levels of plasminogen activator and DNA synthesis (Sato and Rifkin, 1988). It is also believed that insulin-like growth factor-II (IGF-II) can act in an autocrine manner but that it must act with other regulatory factors in order to be effective (Nagarajan et al., 1985). It is difficult to prove that a growth factor can elicit an autocrine response since prolonged exposure of a cell to a growth factor often results in a reduction of functional receptors for that growth factor on the cell surface. This reduction can be brought about by endocytosis of the receptor which is then either eventually re-cycled back to the cell surface or degraded in the lysosomes. Alternatively, the receptors can remain on the cell surface but be changed so they are unable to bind the ligand.

The autocrine growth stimulation hypothesis states that cells can gain growth autonomy by producing, secreting, and responding to a growth factor that is normally supplied exogenously (Sporn and Todaro, 1980). Given this definition, the fact that MRP/PLF is a member of the prolactin-

growth hormone family, and the fact that it has been found to be secreted into the medium by minced placentas from 14-day pregnant BALB/c mice (Linzer et al., 1985), it seems reasonable to suggest that it may in certain situations be an autocrine growth factor. It still remains to be shown that it is capable of binding to cell surface receptors and eliciting a response.

1.09 MRP/PLF and the Mannose-6-Phosphate Receptor

The identification of a receptor for MRP/PLF was accomplished by isolating glycosylated MRP/PLF from the medium of a CHO line that expresses mouse MRP/PLF cDNA, iodinating it and looking for specific binding to membrane preparations of fetal and maternal liver and placenta (Lee and Nathans, 1988). Non-glycosylated MRP/PLF could not compete for the binding sites of the iodinated glycosylated MRP/PLF. D-mannose 6-phosphate was able to inhibit the binding of MRP/PLF to the membranes. The MRP/PLF receptor was bound to an affinity column and eluted with mannose 6-phosphate. It could be specifically immunoprecipitated with antibodies directed against the mannose 6-phosphate receptor and therefore it was concluded that the MRP/PLF receptor is the mannose 6-phosphate receptor or closely related to it. There is no evidence though, that binding to this receptor is involved in MRP/PLF activity. Indeed, preliminary experiments suggest that in contrast to MRP/PLF secreted by CHO cells, MRP/PLF secreted from placenta binds

poorly to the mannose 6-phosphate receptor.

The mannose 6-phosphate receptor circulates between the Golgi apparatus and an acidified pre-lysosomal compartment, acting as a carrier protein in the targeting of acid hydrolases to the lysosomes (von Figura and Hasilik, 1981). Examples of acid hydrolases which are known to bind to the mannose 6-phosphate receptor include a 52-KD estrogen-induced protein secreted by MCF7 cells (Capony et al., 1987), uteroferrin, the major progesterone-induced secretory protein of the porcine endometrium (Baumbach et al., 1984) and the major excreted protein (MEP) of transformed mouse fibroblasts (Sahagian and Gottesman, 1982). Recently it has been shown that the IGF-II receptor appears to be identical to the cation-independent mannose 6-phosphate receptor (Morgan et al., 1987). The significance of the binding of a growth factor and secreted proteins carrying mannose 6-phosphate to the same receptor is not understood at present. This receptor may have several functions since the precursor form of TGF-β has also been shown to bind to it via mannose 6-phosphate (Purchio et al., 1988).

That MRP/PLF binds to a lysosomal targeting protein should not come as a surprise when some of Nilsen-Hamilton's earlier observations are considered. She found that when Swiss 3T3 cells were treated with ammonium chloride or monensin, the amount of extracellular MRP/PLF was increased (Nilsen-Hamilton et al., 1981). These agents have no effect on or cause a decrease in the secretion of

most secretory proteins. In contrast, the secretion of lysosomal proteins is increased by them. MRP/PLF however appears to be a secretory rather than a lysosomal protein since in pulse chase experiments, most of the newly synthesized MRP/PLF was secreted within two hours of its synthesis. Lysosomal proteins are usually retained inside the cells for much longer periods of time after their synthesis (Nilsen-Hamilton et al., 1988).

Recently it has been proposed that crinophagy is involved in the rapid removal of MRP/PLF from the placenta between days 13 and 14 of development (Nilsen-Hamilton et al., 1988). Crinophagy involves the fusion of secretory granules with lysosomes resulting in the degradation of their contents and has been shown to regulate the secreted level of PRL. While there is a secretory stimulus such as thyrotropin releasing factor, the PRL-containing vesicles fuse with the plasma membrane. Upon removal of the stimulus, such as at the end of lactation, the PRL-containing vesicles fuse with the lysosomes resulting in the degradation rather than the release of their contents. It is interesting that it has been proposed that uteroferrin, another protein which binds to the mannose 6-phosphate receptor, may in the uterus represent an overproduced lysosomal protein that is transported along a secretory pathway rather than along a lysosomal pathway (Baumbach et al., 1984).

1.10 MRP/PLF Regulation at the Gene Level

At the time this project was undertaken, relatively little was known about the regulation of MRP/PLF at the gene or mRNA level. It was known that the MRP protein was induced in Swiss 3T3 cells by the addition of serum, EGF, or FGF and this induction could be prevented by the addition of the transcriptional inhibitor actinomycin D (Nilsen-Hamilton et al., 1980). This suggested that at least some of the regulation of MRP by these growth factors was occurring at the transcriptional level. Further support for this theory came in 1985 when it was shown that another transcriptional inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), could prevent the induction of MRP mRNA in Swiss 3T3 cells by fetal calf serum (Parfett et al., 1985). These workers also showed that MRP was the same protein as proliferin. Proliferin mRNA levels had been shown to increase in response to serum and PDGF (Linzer and Nathans, 1984). An analysis using the slot blot technique of the MRP/PLF RNAs present in the nuclei of serum stimulated and resting cells showed that higher levels of MRP/PLF RNAs were present in the nuclei of stimulated cells than in those of resting cells. This was attributed to an increased transcriptional activity (Edwards et al., 1985). More recently FGF has been shown to increase MRP/PLF mRNA levels in Swiss 3T3 cells (Nilsen-Hamilton et al., 1987).

Since these initial observations, it has become obvious

that the regulation of MRP/PLF at the pre-translational level is very complex. There are multiple MRP/PLF genes in mouse cells (Linzer and Wilder, 1986, Ann Marie Craig, this laboratory). As two different MRP/PLF mRNA species have been identified, it appears that at least two of these genes are expressed. The 5' regulatory regions of two MRP/PLF genes were isolated and were shown by CAT assays to have different strengths and different degrees of serum inducibility (Linzer and Mordacq, 1987). This again points to the transcriptional level as being important in regulating MRP/PLF expression but it is not known how the promoters relate to the identified mRNA species.

There is some evidence that RNA processing may also be involved in the regulation of MRP/PLF expression. RNA isolated from both the cytoplasm and nuclei of resting and 12 h serum stimulated BALB/c 3T3 cells, was electrophoresed, blotted and the blot was hybridized to probes representing either one of the introns of an MRP/PLF gene or an MRP/PLF cDNA clone. Transcripts were observed in the nucleus of both the resting and stimulated cells although they were incompletely processed and at much lower levels in the resting cells (Linzer and Wilder, 1987). In another example, nuclear run-on analysis indicated that both MEF and 3T3 cells were capable of transcribing MRP/PLF genes (Edwards et al., 1987). As neither MRP/PLF mRNA nor protein had ever been identified in MEF cells, it was suggested that processing, transport, or stability of the

message was regulating MRP/PLF expression in MEF cells.

The expression of MRP/PLF mRNA is very sensitive to the growth conditions of the cells. MRP/PLF mRNA levels varied in Swiss 3T3, BALB/c 3T3 and BNL cell lines when the RNA was prepared from cells growing in medium containing 10% fetal calf serum while the levels were almost the same in these three cell lines when the mRNA was isolated 20 h after replacing the culture medium with fresh medium containing 10% FBS (Parfett et al., 1985). in situ hybridization of serum stimulated cultures of BALB/c 3T3 cells with ^{35}S-labelled antisense MRP/PLF probe revealed that about a third of the cells were expressing high MRP/PLF levels, a third were expressing moderate levels and a third had low or undetectable levels (Linzer and Wilder, 1987).

1.11 The Aim of this Thesis

As explained in section 1.10, there is very limited knowledge about the regulation of MRP/PLF expression at the pre-translational level. One of the aims of this thesis then is to begin to fill that void and gain a greater understanding of the regulation of expression of MRP/PLF at the pre-translational level in cultured cells. As explained in chapter 4, there is evidence that the regulation of expression of MRP/PLF in cultured cells is probably much like the in vivo regulation of its expression in the placenta. Therefore in the case of MRP/PLF, data acquired

by using a cell culture system can be extended to make predictions about its expression in the placenta.

Greater knowledge concerning the regulation of MRP/PLF expression was gained in the following ways.

1.) A full-length MRP/PLF genomic clone was isolated and characterized. Sequence analysis and Southern blotting allowed the intron/exon structure of this gene to be determined. This showed that MRP/PLF is a member of the prolactin-growth hormone family at the gene level as well as the protein level, as had previously been shown. This is the first member of this family in the mouse to be characterized at the gene level.

2.) The start site of transcription was determined by S1 analysis. More than 1000 bp upstream of this start site was sequenced and compared with sequences reported to be important in gene regulation. This allowed the prediction that some compounds and proteins not previously tested for their effect on MRP/PLF gene regulation would be effective. One such compound, dexamethasone, was subsequently shown by Northern blotting to decrease the amount of MRP/PLF mRNA in the cytoplasm. It could not have been predicted by sequence comparisons whether dexamethasone would increase or decrease MRP/PLF expression.

3.) The upstream region encompassing the transcriptional

start site was cloned into a chloramphenicol acetyl transferase expression vector (pSV0cat) and promoter activity was assayed. This revealed that in the BNL cell system used, the promoter was weak but functional. In addition, there appeared to be a negative regulatory element within a defined 65-bp region. Preliminary experiments showed that the addition of TGF-α or vitamin D3 to the system resulted in an increase in the amount of chloramphenicol acetyl transferase activity assayed. Northern blots also revealed that there was an increase in the amount of MRP/PLF mRNA in the cytoplasm of BNL cells treated with TGF-α or vitamin D3. Taken together, these results suggest that TGF-α and vitamin D3 are capable of regulating MRP/PLF expression and at least part of this regulation is occurring at the transcriptional level. Neither of these compounds had previously been identified as affecting MRP/PLF expression. This allowed speculation of how MRP/PLF expression is regulated in the placenta.

4.) Bal-31 exonuclease was used to create a set of nested mutations in the MRP/PLF promoter and these were cloned into pSV0cat. These will allow the future identification of regions in the MRP/PLF promoter which are responsible for the regulation by TGF-α and vitamin D3.

Another aim of this thesis is to determine at which level MRP/PLF expression is regulated as the non-expressing

mortal MEFs are passaged to become an expressing immortal 3T3 line. As explained in greater detail in chapter 5, MRP/PLF expression gives one a handle with which to study the phenomenon of immortalization. The knowledge of how this expression might be evoked will allow the design of experiments which will characterize the regulatory element responsible for the change in MRP/PLF expression and possibly for the immortalization process.

DNase I hypersensitive sites, differences in methylation patterns, processing and transport of MRP/PLF transcripts, were all looked at as possible mechanisms involved in regulating MRP/PLF expression in mortal and immortal mouse cells. The increased expression of MRP/PLF in the 3T3 cells did not appear to be accompanied by major changes in the genome such as amplification of the gene, generation of new DNase I hypersensitive sites or methylation. This agrees with run-on transcription results that showed that MRP/PLF is transcribed to an equal extent in MEF and 3T3 cells (Edwards et al., 1987). However, MRP/PLF transcripts were not detected in either the nucleus or cytoplasm of MEF cells when transcriptional mapping assays were carried out. This ruled out the possibility that the MRP/PLF transcripts were not being processed or transported out of the nucleus of MEF cells and suggested that either the transcript is very unstable in the MEF nucleus or the phenomenon of intragenic pausing is occurring.

CHAPTER 2

MATERIALS AND METHODS

2.01 Enzymes and Reagents

Restriction endonucleases were purchased from Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs, and Pharmacia. The Sequenase kit was from United States Biochemical Corporation. Calf intestinal alkaline phosphatase, T4 polynucleotide kinase and T4 DNA ligase were from Boehringer Mannheim. All were used according to the manufacturer's directions. Deoxyribonuclease I (DNase I) was from Worthington.

All solutions were made with sterile, deionized and glass distilled water. Solutions used for RNA preparation included 100 µl diethyl pyrocarbonate (DEPC) per 100 ml and were autoclaved 20 min.

The [α-^{35}S]dATP (500 Ci/mmole) and [^{14}C]chloramphenicol were from New England Nuclear. All other labelled material was obtained from ICN.

TGF-α was kindly provided by Dr. R. Harkins, Triton Biosciences Inc. β-estradiol and dexamethasone were from Sigma. Acetyl coenzyme A and Whatman PE SIL G plates were purchased from Pharmacia.

2.02 Bacterial Strains

The *Escherichia coli* strains used in this work are detailed in Table 3. *E. coli* RR1 was the strain usually used for propogation of plasmid DNA. *E. coli* JM103 was used to grow M13 bacteriophages while *E. coli* BNN45 was used as the host for lambda phage.

2.03 Media

E. coli RR1 and *E. coli* BNN45 were grown in Luria (L) broth and L-agar (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl) as described (Miller, 1972). The L-broth was routinely supplemented with 0.2% maltose for BNN45 propagation. For large scale lambda DNA preparations, the BNN45 which had been pre-absorbed with lambda phage was grown in LAM medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, 0.12% $MgSO_4$).

JM103 was grown in YT medium (0.8% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl). To ensure that the *E. coli* JM103 had retained the F plasmid, JM103 was periodically streaked out on minimal glucose agar plates (1.5% agar, 1.05% K_2HPO_4, 0.45% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% sodium citrate, 0.02% $MgSO_4$, 0.2% glucose, 5 ng/ml thiamine HCl). Cells which were to be made competent were grown in SOB medium (2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$). Where necessary, carbenicillin was added at 100 µg/ml.

Table 3

Bacterial Strains

Bacterial Strain	Genotype	Source
JM103	[$^{\Delta}$(lac-pro) rspL endA SbcB15 hsdR4 supE /F' traD36 proAB lacI Z$^{\Delta}$M15]	J. Messing
RRI	F$^-$ hsdS20 (r_B^-,m_B^-) ara-14 proA2 lacYI galK2 rpsL20 (Sm^r) xyl-5mtl-lsupE44,lambda-	F.Bolivar
BNN45	hsdR$^-$ hsdM$^+$ supE44 supF thi met lacY	ATCC

2.04 *Escherichia coli* Stocks

E. coli strains harbouring the appropriate plasmid were inoculated into the desired medium and allowed to grow at 37°C for 18 h. An equal volume of this culture was added to 1 ml of 100% glycerol. These stocks of *E. coli* were stored at -70°C.

2.05 Phage Stocks

Lambda phage stocks were grown, stored, and titrated as described (Schlief and Wensink, 1981). In order to prepare M13 phage stocks, JM103 cells were grown in YT medium until they reached a density of 1 x 10^8 cells/ml at which point the cells are growing logarithmically. M13 phage was added and the infected culture was left at 37°C overnight in a roller drum. One ml of the culture was centrifuged for 5 min in an Eppendorf centrifuge and the supernatant containing the M13 phage was heated for 10 min at 65°C. Usually 10^{11} p.f.u./ml were obtained.

2.06 Genomic Libraries

The lambda 9090 library was obtained from Dr V. Morris. This library was constructed from DNA obtained from BALB/c mouse embryos. It was partially digested with *Sau*3A and *Eco*RI linkers were added. The DNA with linkers was cloned into the *Eco*RI site of the bacteriophage lambda vector Charon 4A (Blattner *et al.*, 1977).

The lambda MEF amplified pool number 4 library was

prepared in this laboratory by Rebecca St. Pierre. Swiss mouse embryo fibroblast DNA which had been partially cleaved with BamHI, BglII, BclI or MboI was cloned into the BamHI site of lambda bacteriophage L47.1 (Loenen and Brammar, 1980).

2.07 Subcloning Vectors

M13mp18 and M13mp19 (Yanisch-Perron et al., 1985), pBR322 (Bolivar et al., 1977), pSP64 and pSP65 (Melton et al.,1984) and the pGEM vectors (Promega Biotec) were all used as recipients in the subcloning of pieces of DNA. Other plasmids used as a source of various DNA fragments are detailed in Table 4.

2.08 Agarose Gels

For analytical purposes the agarose gels usually contained 1% agarose (Seakem) for plasmids and 0.4% agarose for lambda phage and genomic DNA. The agarose was dissolved in running buffer (50 mM TrisHCl (pH 8.0), 5 mM CH_3COONa, 0.5 mM EDTA, 0.5 µg/ml ethidium bromide). When only small amounts of DNA was present, it was visualized by photographing the gel under short wave ultraviolet light (Maniatis et al., 1982).

2.09 Polyacrylamide Gels

Five percent polyacrylamide gels cross-linked with N,N'-methyl-bis-acrylamide (Bio-Rad) were electrophoresed

Table 4

Description of the Plasmids Used in this Work

Plasmid	Source	Significant Features
pBR322	F.Bolivar	Amp^R, Tc^R
pSP65	D.A.Melton	Amp^R, polyclonal restriction site, SP6 promoter
pSP64	" "	" "
pGEM3	Promega Biotec	Amp^R, polyclonal restriction site, SP6 and T7 promoters
pGEM4	" "	" "
E21	P.Waterhouse, this lab.	full length MRP cDNA from BNL cells in pW7
pES2	this work	2-Kbp EcoRI-StuI fragment of mrp/plf1 encompassing exon 1 and 1.1 Kbp of 5' upstream region, subcloned into the EcoRI/PvuII sites of pBR322
p1.9Eco	this work	1.9-Kbp EcoRI fragment of mrp/plf1 encompassing exons 4 and 5, subcloned into the EcoRI site of pBR322
pES6	this work	approximately 6-Kbp StuI-

Table 4 cont'd.

Plasmid	Source	Significant Features
		EcoRI fragment of mrp/plf1 encompassing exons 2 and 3 subcloned into the EcoRI-PvuII sites of pBR322
p4.4Eco	this work	9.6-Kbp EcoRI fragment from MRP/PLF lambda genomic clone 4.4, subcloned into the EcoRI site of pBR322; this clone has some lambda DNA
p2.1Eco	this work	8.4-Kbp EcoRI fragment from MRP/PLF genomic clone 2.1, subcloned into the EcoRI site of pBR322
p1.3Pvu	this work	6-Kbp PvuII fragment from MRP/PLF lambda genomic clone 1.3, subcloned into the PvuII site of pBR322
p4.4PE	this work	1.1-Kbp EcoRI-PstI fragfragment of lambda genomic clone 4.4 cloned into the EcoRI/PstI sites of pSP65; contains part of first exon and 5' flanking sequences

Table 4 cont'd.

Plasmid	Source	Significant Features
p1.3PE	this work	as for p4.4PE except from lambda genomic clone 1.3
p2.1PE	this work	as for p4.4PE except from lambda genomic clone 2.1
pSV0cat	C.Gorman	promotorless plasmid for CAT expression assays
pSV2cat	C.Gorman	plasmid containing the SV40 promoter to drive cat gene expression
pEPcat	this work	1.1-Kbp EcoRI-PstI fragment containing part of the first exon and 5' flanking sequences of mrp/plf1 inserted into pSV0cat
p2Pcat	this work	2-Kbp PstI fragment containing part of the first exon and about 2 Kbp of 5' flanking sequences from p1.3Pvu inserted into pSV0cat
pSPcat	this work	1.05-Kbp ScaI-PstI fragment from pEPcat inserted into pSV0cat; the putative negative regulatory ele-

Table 4 cont'd.

Plasmid	Source	Significant Features
		ment has been deleted in this clone
pP5	this work	pSP65, cleaved with SphI and EcoRI and re-ligated; lacks the SP6 promoter
pP5cat	this work	the cat gene, small t splice site and poly A addition site from pSV2cat inserted into the HindIII-PvuII sites of pP5
pP5MRPcat series	this work	members consist of 5' deletions of the mrp/plf1 promoter cloned into the SmaI-PstI sites of pP5cat
PLF42cat	D.Linzer	EcoRI-PstI promoter fragment from PLF42 cloned into pUC-cat
pGMRP2	this work	final 2 exons of mrp/plf1 and surrounding sequences subcloned into the EcoRI site of pGEM3 oriented so antisense RNA transcripts can be generated with the use of T7 RNA polymerase
pPH2GEM3	this work	full length MRP/PLF cDNA

Table 4 cont'd.

Plasmid	Source	Significant Features
		subcloned into the PstI-HindIII sites of pGEM3; use of T7 RNA polymerase yields antisense RNA transcripts

in (50 mM Trisborate (pH 8.3), 1 mM EDTA) buffer. Bromophenol blue dye was used to monitor the extent of the DNA migration. After it had run sufficiently, the gel was stained in 1 µg/ml ethidium bromide for 10 min, destained 5 min in water and was photographed under short wave ultraviolet light (Maniatis et al., 1982).

2.10 Isolation of Specific DNA Fragments

DNA fragments greater than 700 bp were isolated from agarose gels made of low melting point agarose. The progress of the DNA migration was monitored with a hand held long wave UV light source. The band of interest was cut out and the agarose was melted by heating at 70°C for 5 to 10 min. Two volumes of phenol equilibrated with 0.1 M TrisHCl (pH 8.0) which had been pre-warmed to 37°C were added, the mixture was thoroughly vortexed and was left in a 37°C incubator for 10 min. The phases were separated after a 5 min centrifugation at room temperature. The top aqueous phase containing the DNA fragment was removed and the above steps were repeated. The aqueous phase was extracted with phenol/chloroform and finally with chloroform. The DNA was further purified by spermine precipitation (Hoopes and McClure, 1981). One tenth volume of 0.1 M spermine was added to the DNA solution and left on ice 1 h. The DNA was pelleted after a 15 min centrifugation in the microfuge and was rinsed with 70% ethanol. After a 5 min centrifugation, the DNA was re-

suspended in 1 ml spermine extraction buffer (75% ethanol 0.3 M NaCl, 10 mM $(CH_3COO)_2Mg$ and left on ice for 1 h with periodic vortexing. The DNA was finally recovered after a 15 min centrifugation, rinsed with absolute ethanol and re-suspended in TE buffer (10mM TrisHCl (pH 8.0), 1mM EDTA).

DNA fragments smaller than 700 bp were eluted from 5% polyacrylamide gels. After visualization, the band of interest was excised from the gel, cut into small pieces and added to 400 µl of elution solution as described (Maxam and Gilbert, 1980). Elution solution contains (0.5 M CH_3COONH_4, 10 mM $(CH_3COO)_2Mg$, 1 mM EDTA, 0.1% SDS). The extraction was allowed to proceed for 18 h at 32°C in a rotating drum at which point a short centrifugation was given, the supernatant was retained, and the gel pieces were further extracted with 200 µl elution solution. Three hours later, this latter supernatant was collected and combined with the first supernatant. They were extracted with phenol/chloroform, then with chloroform and were ethanol precipitated.

2.11 Nick-Translation of DNA

The nick-translation procedure (Rigby, 1977) was used in order to synthesize ^{32}P-labelled DNA as a probe in plaque screening, Southern blot analysis and colony screening. One hundred nanograms of purified DNA fragment to be used as probe (section 2.10) was resuspended in buffer (50 mM TrisHCl (pH 8.0), 5 mM $MgCl_2$) containing all

four deoxynucleoside triphosphates (dNTP) at least two of which were labelled with ^{32}P at the α position. This mixture was incubated with 2.5 µg/ml DNase I (Worthington) for 3 min at room temperature. One-to-two units of E. coli DNA polymerase I was added and the reaction was left for 2 h at 15°C. The reaction was terminated by adding EDTA to 20 mM and heating the solution at 65°C for 5 min. Unincorporated dNTPs were removed by passing the solution through a Sephadex G-50 (medium) column. DNA was usually labelled to a specific activity of 1 x 10^8 cpm per microgram.

2.12 Pre-Hybridization, Hybridization, and Washing of Nitrocellulose Filters

Nitrocellulose filters which had picked up DNA after plaque screening, Southern blot transfer or colony screening were dried under a heat lamp and baked for 2 h at 80°C. They were placed in a Kapak bag and pre-hybridized in hybridization solution (50% formamide, 5X SSC, 10X Denhardts solution, 100 µg/ml salmon sperm DNA) for at least 4 h at 42°C. SSC is (0.15 M NaCl, 0.015 M sodium citrate). Approximately 1 x 10^6 cpm of nick translated probe was boiled for 5 min and added in hybridization solution to the pre-hybridized filters. The hybridization was continued for another 16 to 20 h at 42°C. Following removal of the probe, the filters were given 3 15-min washes at 42°C in 2X SSC, 0.1% SDS. They were then washed two times in 0.2X SSC, 0.1% SDS for 20 min each time, again

at 42°C. The filters were air dried and exposed to Kodak XAR-5 film with an intensifying screen at -70°C for autoradiography. When it was necessary to totally remove a probe, blots were heated in pre-hybridization buffer at 75°C for 10 min.

2.13 Screening Lambda Genomic Libraries by Plaque Lifts

The host cells, BNN45, were grown to log phase in L-broth containing 0.2% maltose. They were centrifuged at 1500 rpm for 10 min at 4°C and were resuspended in an equal volume of 10 mM $MgSO_4$.

On the first screen, 4 x 10^4 phage from either the lambda MEF amplified pool number 4 or the 9090 library were combined with 100 µl of the host cell culture described above and were incubated at 37°C for 15 min to allow pre-absorption to occur. After pre-absorption, they were mixed with 7 ml of molten 0.7% tryptone top agarose and plated on 150 mm 0.2% glucose/agarose plates which had been pre-warmed to 37°C. The plates were incubated for about 11 h at 37°C in an inverted position prior to being transferred to 4°C for 1 h. Nitrocellulose filters were gently placed on the plates. A needle was used to stab through the nitrocellulose and agar in 3 different places so that the corresponding plates and filters could later be re-aligned. After the filters had become evenly wetted, they were peeled off the plates and were trans- ferred DNA side up onto Whatman 3MM paper soaked in (1.5 M NaCl, 0.5 M

NaOH) where they remained 5 min. The filters were then transferred DNA side up onto Whatman 3MM paper soaked in (1.5 M NaCl, 0.5 M TrisHCl (pH 8.0)) and were again left for 5 min. Finally they were transferred DNA side up onto Whatman 3MM paper soaked in 2X SSC, left for 5 min and treated as described in section 2.12.

The probe used was nick-translated full length MRP/PLF cDNA known as E21 which was isolated by Paul Waterhouse from the library previously described (Edwards et al., 1986).

Following re-alignment of filters, X-ray film and plates, positive areas were picked into 1 ml SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM TrisHCl (pH 7.5), 0.01% gelatin), left on ice for several hours and spot titrated.

The second screen was identical to the first except that only 1 x 10^4 phage were applied per plate. Small plates were employed for the third and subsequent screens. On the third screen, 1 x 10^3 phage were mixed with 50 µl of host cell culture and 3.5 ml of top agarose. The fourth screen was used to ensure that plaques picked from the third screen were indeed pure so only 1 x 10^2 phage were plated.

2.14 Small Scale Lambda DNA Preparations

In order to obtain enough lambda DNA for several restriction digestions, the plate lysate procedure (Maniatis et al., 1982), was used with the following

modifications. Approximately 5 x 10^8 purified phage were mixed with 200 µl BNN45 and were pre-absorbed and plated on LB/0.2% glucose plus 1.5% agarose as described (section 2.13)

The pellet obtained from the isopropanol precipitation was washed with 70% ethanol, 0.1 M CH_3COONa. It was dried briefly and resuspended in 50 µl TE buffer. Five microliters of 0.1 M spermine was added and left 30 min on ice. The sample was centrifuged 15 min and the pellet was rinsed with 70% ethanol. Spermine extraction buffer (section 2.10) was added and the pellets were left for about 1 h on ice with occasional mixing. Finally they were centrifuged for 15 min, washed with absolute ethanol, dried briefly and resuspended in 50 µl TE buffer. All restriction digestions done on DNA prepared in this manner included 5 µl of DNA and the addition of DNase-free pancreatic RNase (20 µg/ml).

2.15 Large Scale Lambda DNA Preparations

A modification of a published procedure (Frischauf et al., 1983) was used for large scale lambda DNA preparations. E. coli BNN45 cells were grown in LB/0.2% maltose with good aeration until the density reached 2 x 10^8 cells/ml. They were pelleted, resuspended in SM buffer, and infected with high titre lambda phage stock (m.o.i 0.15). They were added to LAM buffer and were left in a shaking incubator at 37°C for 16 h. Chloroform (1/150

volume) was added and the cultures were left shaking an additional 15 min. Bacterial debris was removed by centrifugation at 5000 rpm for 15 min. Ribonuclease A and DNase I were both added to the supernatant at 1 µg/ml and this was left at 4°C for 1 h. The solution was adjusted to 5.75% NaCl and 10% polyethylene glycol-8000 and was left on ice for 4 h at which time it was again centrifuged 5000 rpm for 15 min. The pellet was resuspended in 1/80 the original culture volume CsCl/SM buffer (density 1.5 g/ml) and was centrifuged in a SW50.1 rotor 20 h, 35,000 rpm, at 15°C. The virus band was collected and 3 volumes of water and 8 volumes of ethanol were added. It was stored overnight at -20°C and was centrifuged at 7500 rpm for 15 min. The pellet was dried, resuspended in 50 mM TrisHCl (pH 8.0) and incubated for 1 h at 37°C with 50 µg/ml proteinase K. The DNA was extracted with phenol/chloroform, chloroform and was precipitated by the addition of 1/4 volume of 10 M ammonium acetate and 2 volumes of ethanol.

2.16 Southern Blot Analysis

In order to map restriction sites in the genomic clones, Southern blot analysis was performed. Restricted DNA was run on an agarose gel as described in section 2.08. The gel was soaked in 0.25 M HCl for 15 min. The DNA was denatured by soaking the gel twice in (1.5 M NaCl, 0.5 M NaOH) for 15 min each time. Finally it was neutralized by soaking in (0.5 M TrisHCl (pH 8.0), 1.5 M NaCl), twice, 15

min each time. The DNA was transferred to nitrocellulose as described (Southern, 1975), in 20X SSC buffer overnight. The blot was treated as described in section 2.12.

2.17 *In Vitro* Production of Blunt-Ended DNA

DNA was restricted and then treated by one of the following methods: Recessed 3' ends were incubated in (50 mM TrisHCl (pH 7.6), 10 mM $MgCl_2$) in the presence of the four deoxynucleoside triphosphates (at 0.5 mM) and Klenow fragment (1-to-5 U) for 15 min at 25°C (Maniatis *et al.*, 1982).

Mung bean nuclease was the method of choice for removing 5' and 3' overhangs. The DNA sample was resuspended in buffer (30 mM CH_3COONa (pH 4.6), 50 mM NaCl, 0.5 mM $ZnSO_4$, 5% glycerol) at a final concentration of 50 µg/ml. Twenty units of mung bean nuclease was added per µg DNA and the mixture was incubated at 30°C for 20 min. The reaction was terminated by the addition of EDTA to 25 mM, followed by extraction with phenol/chloroform and then chloroform. The DNA was recovered by ethanol precipitation.

An alternative method for the removal of 3' and 5' overhangs involved the use of S1 nuclease. One microgram of DNA was added to 50 µl of S1 nuclease buffer (30 mM CH_3COONa (pH 4.5), 3 mM $ZnCl_2$, 0.3 mM NaCl). Six units of S1 nuclease was added and the mixture was left at room temperature for 15 min. EDTA was added to 20 mM in order to stop the reaction then phenol/chloroform and chloroform

extractions were carried out. Finally an ethanol precipitation was performed in order to recover the DNA.

2.18 Bal-31 Deletions

Bal-31 exonuclease (New England Biolabs) was used to prepare a series of overlapping deleted fragments covering the area of interest. The fragment of interest was cloned into a vector such as pSP64 or pSP65 which carries a multicloning site. A restriction cut was made in the vector near the end of the fragment from which the deletions were to start. The cleaved plasmid DNA was placed in Bal-31 buffer (600 mM NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 20 mM TrisHCl (pH 8.0), 1 mM EDTA) and approximately 1 U of Bal-31 was added per 20 µg DNA. The reaction was incubated in a 30°C water bath and aliquots were removed every 5 min for 30 min. The reaction was terminated by adding EGTA (pH 8.0) to 50 mM and quick freezing in dry ice. After an ethanol precipitation, the ends were blunted with Klenow (2.17) and a second restriction digestion was carried out to release the deleted inserts from plasmid DNA.

2.19 Dephosphorylation

A published procedure (Barker et al., 1983) was employed to dephosphorylate DNA fragments. Cleaved DNA was resuspended in 50 µl 0.1 M TrisHCl (pH 8.4). Two units of calf intestinal alkaline phosphatase was added and the reaction was left for 30 min at 55°C. Following 3 phenol-

chloroform and 1 chloroform extractions, the DNA was ethanol precipitated.

2.20 Ligations

DNA to be ligated was quantified by electrophoresing a sample of the test DNA and known amounts of standard DNA in an agarose gel and comparing band intensities after ethidium bromide staining. Overnight ligations were set up with 100 ng of dephosphorylated vector and a 10X molar amount of insert in a 10 µl volume of ligation buffer (50 mM TrisHCl (pH 8.0), 10 mM $MgCl_2$, 20 mM DTT, 10 mM ATP). One unit of T4 DNA ligase was added and the reaction was left at 15°C (Maniatis et al., 1982).

2.21 Transformations

Transformations were performed as described (Hanahan, 1983), with the exception that after heat shock, 1 ml of normal growth medium was added directly to the competent cells and they were incubated 2 h at 37°C without agitation. Cells were grown on agar plates containing an appropriate antibiotic that allowed the selection of transformed cells having the corresponding antibiotic resistance gene (ampicillin or tetracycline) carried by the introduced plasmid.

JM103 was transformed with M13 in the same manner except that after the heat shock step, the cells were left for 5 min on ice and then they were immediately plated.

The plating procedure involved adding aliquots of competent cells to 2.5 ml molten top agar (0.7% agar, YT medium), freshly supplemented with 10 µl 0.1 M IPTG, 40 µl 2% X-gal and some growing JM103 to form a bacterial lawn for phage growth. The plates were incubated at 37°C overnight. White plaques indicating the presence of an inserted piece of DNA in the β-galactosidase gene carried by the phage were picked for further analysis.

2.22 Alkaline Extraction

The alkaline extraction procedure was used to quickly confirm the presence or absence of a plasmid in a clonal population. The cells from 1 ml of an overnight culture were pelleted for 5 min in a microfuge. The pellet was resuspended in 300 µl of cracking buffer (50 mM TrisHCl (pH 7.6), 1% SDS, 2 mM EDTA, 0.4 mM sucrose, 0.1% bromophenol blue), mixed and left at room temperature for 30 min. After a 15 min centrifugation, 30 µl of the supernatant was removed and loaded onto an agarose gel (Maniatis et al., 1982).

2.23 Colony Screening

The method of colony screening was used when there was a high background of bacterial colonies not containing the desired recombinant vector compared to those carrying the recombinant vector. A nitrocellulose circle was placed on the plate thereby allowing partial transfer of each colony

to the nitrocellulose. After marking the relative positions of the nitrocellulose and the plate, the nitrocellulose was transferred colony side up to Whatman 3MM paper soaked in 0.5 M NaOH. After 5 min the nitrocellulose was transferred to a fresh sheet of 3MM paper soaked in (1.5M NaCl, 0.5 M TrisHCl (pH 7.4)). Five minutes later the nitrocellulose was transferred to 3MM paper soaked in (1.5 M NaCl, 0.03 M sodium citrate) where it remained for 5 min. They were baked for 2 h at 80°C under vacuum. The baked filters were floated on the surface of 6X SSC until they were thoughly wetted from beneath at which point they were submerged for 5 min. They were then pre-washed at 42°C for 1-to-2 h in a solution containing (50 mM TrisHCl (pH 8.0), 1 mM EDTA, 0.1% SDS). They were pre-hybridized and hybridized as described in section 2.12.

2.24 RF1 and Plasmid DNA Preparation

Small scale plasmid preparations (1.5 ml of bacterial culture) were obtained by the boiling method (Maniatis *et al.*, 1982). Phenol/chloroform and chloroform extractions were done prior to the ethanol precipitation step.

Small scale RF1 M13 preparations were done in much the same way. JM103 was first grown to a cell density of 1 x 10^8 cells/ml then infected with M13 phage (m.o.i. of 20) and left to grow for 2½ h at 37°C. Following this they were treated as a boiling preparation for plasmid

extraction.

For large scale preparations, plasmid DNA was purified from a lysozyme-Brij 58 cleared lysate by isopycnic centrifugation in CsCl-ethidium bromide. The DNA was extracted with n-butanol, ethanol precipitated, phenol/chloroform extracted, chloroform extracted and alcohol precipitated a second time (Maniatis et al., 1982). When the DNA was to be used for transfecting mammalian cells, it was isolated after two CsCl gradient centrifugations. The same procedure was used to prepare M13 RF1 DNA with the following modifications. JM103 was inoculated into YT medium and grown to a density of 1 x 10^8 cells/ml at which time they were infected with M13 phage. After 2½ to 3 h, they were treated as a plasmid preparation except that it was necessary to do two CsCl gradient centrifugations to minimize contamination of RF1 with single stranded DNA.

2.25 Complementation (C) Tests

The C test was used to identify M13 clones carrying inserts in the opposite orientation to the parental clone. Thirty microlitres of the parental high titre phage stock, 30 µl of the test high titre phage stock, 3 µl of 0.5 M EDTA and 1.5 µl of 10% SDS were mixed, incubated at 75°C for 1 h and dried down to a volume suitable for loading on an agarose gel. Clones containing complementary inserts hybridize and thus were identified by virtue of their slower mobility on an agarose gel (Messing, 1983).

2.26 Preparation of Single-Stranded DNA

Single-stranded DNA to be used for sequencing reactions was prepared according to Messing (1983), as modified by G. Mackie (personal communication).

JM103 cells, growing in YT medium were infected with high titre M13 phage stock (m.o.i. of 20) when a density of 1 x 10^8 cells/ml was attained. The infected cells were agitated at 37°C for 5½ to 7h at which point 1.5 ml of the culture was centrifuged for 5 min at 10,000 g. To 1.2 ml of the supernatant, 300 µl of (25% PEG-8000, 2.5 M NaCl) was added and the mixture was left at room temperature for 15 min then on ice for 5 min. The samples were centrifuged for 15 min and the resulting precipitate was resuspended in 800 µl (10 mM TrisHCl (pH 8.0), 1 mM EDTA, 0.5% sarcosyl). The samples were reprecipitated with 200 µl of the PEG solution as above, resuspended in 200 µl (10 mM TrisHCl (pH 8.0), 1 mM EDTA, 0.5% sarcosyl) and extracted sequentially with phenol equilibrated with 0.1 M TrisHCl (pH 8.0), phenol/chloroform equilibrated with 0.1 M TrisHCl (pH 8.0) and chloroform:isoamyl alcohol (24:1). The DNA was recovered by ethanol precipitation, resuspended in 45 µl TE buffer containing 10 mM $MgCl_2$ and reprecipitated with 10 mM spermine (see section 2.10). The final precipitate was resuspended in 15 µl TE and was ready to be used in sequencing reactions.

2.27 DNA Sequencing

Restriction fragments of the appropriate regions of the MRP/PLF clones were subcloned into M13mp18 and mp19. These M13 MRP/PLF subclones provided the source of single-stranded DNA for sequencing (section 2.26). Sequencing was done using the Sequenase kit (United States Biochemicals) according to the manufacturer's directions with the following modifications. Usually the labelling reaction was divided in half after 2 min. One half was immediately subjected to the termination reaction while the other half was allowed to continue labelling for another 6 min to give an 8 min labelling reaction. Occasionally, the dGTP or dITP labelling mixture was not diluted when it was desired to read long sequences. The 8 min labelling reactions were always loaded on the gel at least 2 h before the 2 min labelling reactions. The amount of [α-^{35}S]dATP (10 µCi/µl) used in each reaction was increased from 0.5 µl to 1.0 µl.

2.28 Double-stranded DNA sequencing

Double-stranded sequencing (Zhang *et al.*, 1988) was used to sequence across the boundary between the *cat* gene and the MRP/PLF promoter in the clones described in sections 4.06 and 4.07. A solution of 3 µg of plasmid DNA purified on a CsCl gradient was made (0.2 M NaOH, 0.2 mM EDTA) and incubated at room temperature for 5 min. It was neutralized by adding $CH_3COO(NH_4)$ (pH 4.6) to 0.2 M and 3 volumes of CH_3CH_2OH. The reaction was left to precipitate

on ice for 10 min before being centrifuged in a microfuge for 30 min. The pellet was rinsed with 70% ethanol, dried and was used immediately for sequencing using the Sequenase kit described in section 2.27. A primer that anneals to the *cat* gene was used instead of the universal primer.

2.29 Cat Primer

An oligodeoxynucleotide primer was synthesized by Dr. G. Mackie that will bind approximately 50 bp downstream of the *cat* gene and allow one to sequence across the junction of the inserted promoter and the *cat* gene. The sequence of the primer is 5' CGGTGGTATATCCAGTG 3'. When performing double-stranded sequencing, 5 ng of this primer was annealed to 3 µg plasmid DNA.

2.30 Sequencing Gels

Tapered spacers were used to create "wedge" gels that improve the overall resolution and the number of bases that can be read in a single run. A "sharks tooth comb" was used to maximize the number of lanes. Gels were loaded in the order ACGATCGT. Such a pattern aids the eventual reading of the gel since each base is next to all the other bases. Samples were electrophoresed at 35 mA on an 8% acrylamide gel, cross-linked with N,N'-methylene bis-acrylamide (Bio-Rad) in (50 mM Trisborate (pH 8.3), 1 mM EDTA, 7 M urea) until the bromophenol blue tracking dye just ran off the end of the gel. The gel was fixed in

(7.5% acetic acid, 5% methanol), dried under vacuum and heat onto Whatman 3MM paper and exposed to XAR-5 film for autoradiography.

2.31 Analysis of Sequence

Sequences were analysed by the programmes of Mount and Conrad, (1986), or Wilbur and Lipman, (1983), on an IBM AT computer.

2.32 5'-Labelling of DNA with T4 Polynucleotide Kinase

DNA 5' end-labelled with ^{32}P was generated by the method of Barker et al., (1983). Ten micrograms of cleaved DNA was dephosphorylated (section 2.19), resuspended in 30 µl denaturation buffer (10 mM TrisHCl (pH 9.5), 0.5 mM spermidine, 0.05 mM EDTA), and heated to 70°C for 5 min. It was placed on ice and the buffer was adjusted to (50 mM TrisHCl (pH 9.5), 10 mM $MgCl_2$, 5 mM DTT and 5% glycerol). When the end to be labelled was recessed (such as a PstI end), 5% PEG was also included. One hundred µCi of (gamma-^{32}P)dATP and 10 U of polynucleotide kinase were added. The reaction was left at 45°C for 30 min. In those cases where PEG had been added to the reaction, two chloroform extractions were performed to remove some of the PEG before the reaction was stopped by rendering it 0.2 M CH_3COONH_4. The volume was adjusted to 100 µl and applied to a Sephadex G-50 (coarse) column prepared in a 1 cc syringe. Following centrifugation at

2500 rpm, the DNA was present in the flow-through fraction while the unincorporated nucleotides remained on the column (Maniatis et al., 1982).

2.33 S1 Mapping the Site of MRP/PLF mRNA Transcription Initiation

pES2 (Table 4) was restricted with PstI generating two fragments. The DNA was 5' end-labelled at the PstI restriction site using the kinase reaction (section 2.32). It was subsequently cleaved with EcoRI so that the fragment of interest had only one labelled end. Approximately 50 ng of this fragment, with a specific activity of 4.8×10^8 cpm/µg, was added to 10 µg of poly(A)$^+$ selected RNA obtained from BNL cells which had been treated for 20 h with 10 ng/ml TPA. The nucleic acids were suspended in 30 µl hybridization buffer (80% formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.5), 1 mM EDTA) and sealed in a glass capillary tube. They were heated for 15 min in a 70°C water bath and left at 37°C overnight. The next day the hybridization reactions were adjusted to 400 µl with chilled S1 nuclease buffer (0.03 M CH_3COONa (pH 4.5), 0.25 M NaCl, 1 mM $Zn3O_4$, 5% glycerol, 20 µg/ml denatured calf thymus DNA) containing 40 U S1 nuclease (Calbiochem). Samples were incubated at 37°C for 45 min and were ethanol precipitated using 5 µg carrier DNA (Weaver and Weissmann, 1979). The DNA was resuspended in formamide stop mix (95% deionized formamide, 10 mM EDTA, 0.5% bromophenol blue and 0.5% xylene cyanol)

and electrophoresed on a sequencing type gel (section 2.30), except that the wedge-shaped spacers were not used.

2.34 Mammalian Cell Culture

All cell lines were stored as frozen stocks in liquid nitrogen. Cells were grown in the medium as indicated at 37°C in 5% CO_2. The mouse embryo fibroblast (MEF), 3T3, and 3T12 cell lines were derived in our laboratory from Swiss mice by published procedures (Todaro and Green, 1963). They were maintained in Dulbecco's modified Eagles medium (DMEM), supplemented with penicillin, streptomycin, sodium pyruvate and 10% calf serum (Gibco). The 3T3 cells were passaged every 3 days at 1.5 x 10^4 cells/cm^2 while the 3T12 cells were passaged every 3 days at 6 x 10^4 cells/cm^2.

The BNL cell line derived from neonatal BALB/c mouse liver (Patek et al., 1978) was obtained from American Type Culture Collection (ATCC). It was passaged every 3 days at 7.5 x 10^5 cells/100 mm plate and maintained in DMEM supplemented with penicillin, streptomycin, sodium pyruvate and 10% fetal calf serum.

2.35 Transfection of Cells

The calcium phosphate-DNA co-precipitation technique was used for introducing DNA into mammalian cells for the purpose of transient expression assays. The cells were seeded the day before transfection at a density so that they would be in the logarithmic phase of growth at the

time of harvest. MEF and 3T3 cells were plated at 3 x 10^5 cells per 100 mm dish while BNL cells were plated at 7.5 x 10^5 cells per 100 mm dish.

On the day of transfection, a 10X Hepes-buffered saline (HBS) solution was diluted to 2X HBS and the pH was brought to 7.1 with NaOH (1X HBS is 21 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM $NaHPO_4$, 5.6 mM glucose).

A pre-mix containing (250 mM $CaCl_2$, 10 mM NaCl) was combined with gradient purified DNA (section 2.24). The DNA solution was added dropwise to an equal volume of 2X HBS. Nitrogen gas was bubbled through the HBS solution to ensure rapid mixing. The solution was left at room temperature for 45 min to allow further development of the precipitate. Four hundred and sixty µl was applied to each 100 mm dish so that each received 10 µg DNA and the precipitate was left on the cells for 6 h. The precipitate and medium were gently aspirated and the cells were fed with the appropriate growth medium.

2.36 Preparation of Cell Lysates

Cell lysates were prepared from transfected cells 40 h after the precipitate was removed. The cells were kept on ice and cold PBS was used at all times during harvesting. The medium was aspirated from the cells, and they were rinsed with 5 ml PBS. The cells were scraped into 3 ml PBS with a rubber policeman and placed in a conical tube; the plates were rinsed with an additional 3 ml PBS which was

added to the conical tube containing the cells harvested from that plate. Cells were centrifuged at 1000 rpm for 5 min. The supernatant was gently poured off the cellular pellet, which was allowed to drain thoroughly. Finally the cell pellet was resuspended in 100 µl 0.25 M TrisHCl (pH 7.8), transferred to a 1.5 ml Eppendorf tube and was quickly frozen in a dry ice/ethanol bath. It was transferred to a 37°C water bath, thawed, and vortexed vigourously. The freeze/thaw cycle was repeated an additional 2 times. Cell debris was removed by centrifugation for 15 min at 4°C.

2.37 Bradford Assay for Protein Determination

The Biorad Bradford protein determination kit was used to determine the protein concentration of cellular lysates. A standard curve of 1 to 20 µg/ml BSA in water was obtained. An appropriate amount of cell lysate was assayed so it fell in the linear range of the standard curve.

2.38 CAT Assay

The assay for chloramphenicol acetyl transferase (CAT) activity was described (Gorman *et al.*, 1982). Fifty micrograms of protein as determined by the Bradford assay was combined with 0.25 M TrisHCl (pH 7.5) in a final volume of 120 µl. To this was added 60 µl pre-mix containing (1.3 mM acetyl CoA and 0.15 µCi [^{14}C]chloramphenicol). The reaction progressed at 37°C for 2 h, and was terminated by

the addition of 1 ml ethyl acetate. After vortexing, the phases were separated by a 5 min centrifugation and 900 µl of the upper phase was removed into a clean tube. It was dried down under a stream of nitrogen gas, resuspended in 25 µl ethyl acetate and spotted onto a Whatman PE SIL G plate. The developing solution was chloroform:methanol 19:1. TLC plates were exposed directly to Kodak XAR-5 film for autoradiography. After the film was developed, it was lined up with the TLC plate and radioactive spots on the TLC plates were cut out for quantitation by scintillation counting.

2.39 Isolation of RNA from Mammalian Cells

The cells were trypsinized as usual and washed well with PBS. The cellular pellet (4 x 10^6 cells) was resuspended in 1 ml TSM buffer (10 mM TrisHCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40), incubated 3 min on ice and homogenized with 5 strokes of a Dounce homogenizer. Nuclei were removed by centrifugation at 1500 rpm for 5 min. Nuclear RNA was prepared by suspending the pellet in 1 ml TSM buffer, dripping it into 10 volumes lysis buffer (10 mM TrisHCl (pH 8.0), 7 M urea, 0.35 M NaCl, 1 mM EDTA, 2% SDS) and extracting with phenol/chloroform. A 10,000 rpm, 10 min centrifugation at 20°C separated the layers. The aqueous layer was removed and re-extracted with phenol-chloroform. RNA was precipitated by adding CH_3COONa (pH 5.5) to 0.3 M and 2.5 volumes ethanol. RNA was recovered

by centrifugation for 1 h at 10 K, 4°C. The pellet was dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water. Purity of all RNA samples was assessed by UV scanning from 220 to 320 nm and RNA was quantitated by taking the absorbance reading at 260 nm and subtracting the background absorbance at 320 nm. Cytoplasmic RNA was prepared by dripping the supernatant obtained from the first centrifugation into an equal volume of lysis buffer and treating this as described for nuclear RNA.

2.40 Selection of Poly(A)$^+$ RNA

The RNA obtained above was dissolved in 1 ml binding buffer (10 mM TrisHCl (pH 7.5), 0.5 M NaCl, 0.5% SDS) and heated to 65°C for 5 min. It was chilled and passed over a 2 ml oligo dT cellulose column. The flow through was passed over the column 5 times before 20 ml binding buffer was used to wash the non-bound material off. Poly(A)$^+$ RNA was eluted in 8 ml elution buffer (10 mM TrisHCl (pH 7.5), 0.05% SDS). Ammonium acetate was added to 2.5 M and 2 volumes of ethanol were added to precipitate the RNA (section 2.39).

2.41 Northern Blot Analysis

Northern blot analysis was used to determine the amount of a specific RNA species in a population of RNA molecules. The formaldehyde gels used for Northern blotting contained 1.1% agarose dissolved in MOPS buffer (40 mM MOPS (pH

8.0), 10 mM CH_3COONa, 1 mM NaEDTA) containing 18% formaldehyde. Ten µg of each cytoplasmic RNA sample (section 2.39) was dried, rehydrated in 3 µl DEPC-treated water and combined with 19.5 µl of formamide/formaldehyde mix (65% deionized formamide, 23% formaldehyde in gel buffer). Prior to loading the samples were heated at 55°C for 15 min, placed on ice and combined with 1 µl stop mix (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol). Gels were run with constant buffer recirculation at 50 volts until the bromophenol blue was approximately 2 cm from the bottom of the gel. The RNA was transferred from the gel to GeneScreen Plus (NEN) in 10X SSC buffer overnight using a technique analogous to the Southern blot described in section 2.16, except that the gel was not pretreated. After transfer, the blot was baked for 2 h at 80°C under vacuum and RNA was covalently bound to dry filters by irradiation with 160 J/m^2 delivered by a germicidal lamp.

2.42 Oligolabelling of DNA

The oligolabelling kit (Pharmacia) based on the technique of Feinberg and Volgelstein (1983) was used according to the manufacturer's directions. One hundred ng gel-purified "insert" DNA was labelled for 4 h. Unincorporated dNTPs were removed by passing the solution through a Sephadex G-50 (medium) column.

2.43 Pre-hybridization, Hybridization and Washing of GeneScreen Plus

Pre-hybridization and hybridization of GeneScreen Plus was done according to the manufacturer's directions. Blots were stored in sealed Kapak bags to prevent them from drying out. When it was necessary to totally remove a probe, the blots were heated for 1 h at 90°C in a solution containing (0.5X Denhardt's solution, 25 mM TrisHCl (pH 7.5), 0.1% SDS) and rinsed in distilled water at 65°C for 15 min.

2.44 Isolation of Genomic DNA from Mammalian Cells

The nuclear pellet was obtained from cells as described in section 2.39. The nuclei were washed twice with a solution containing (10 mM TrisHCl (pH 7.4), 50 mM NaCl, 3 mM $MgCl_2$) and centrifuged for 5 min at 2500 rpm after each wash. Five volumes of lysis buffer without SDS were added (0.1 M EDTA, 0.2 M TrisHCl (pH 8.5), 100 µg/ml proteinase K) then five volumes of lysis buffer with 2% SDS were added. The solution was left at 60°C for appproximately 3½ h with swirling every ½ h. Potassium acetate was added to 0.1 M, the mixture was left at 4°C for 30 min and centrifuged 15 min 12,000 rpm. Two volumes of ethanol were added to the supernatant. After being left at -20°C, the DNA was recovered by centrifugation at 5,000 rpm for 30 min and was washed with 70% ethanol and absolute ethanol. The pellet was dried, resuspended in TE buffer and treated with

50 µg/ml RNase A for 1 h at 37°C. Proteins were removed by exhaustive phenol/chloroform extractions. The DNA was concentrated after an ethanol precipitation.

2.45 Analysis of DNA by Methylation Sensitive Enzymes

The isoschizomer pair of enzymes, MspI and HpaII, were used according to the manufacturer's directions. Usually 7.5 U/µg DNA was added and the digestion was allowed to proceed about 6 h before an additional 2.5 U/µg DNA was added. The extent of digestion was monitored by including excess commercial lambda DNA in parallel digestions. After the digestion had gone to completion, as determined by complete lambda digestion, the samples were subjected to phenol/chloroform extractions and ethanol precipitations. DNA was quantitated by its absorbance reading at 260 nm and equal amounts of DNA were analysed by Southern blotting.

2.46 DNase I Studies

Cells were removed from the plates by scraping with a rubber policeman rather than by trypsinization. The nuclear pellet (section 2.39) was resuspended in DNase I digestion buffer (10 mM TrisHCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$) and treated for 5 min at 37°C with various amounts of DNase I such that the final concentration of DNase I in the sample varied from 0 to 10 µg/ml. The reaction was terminated by adding EDTA to 50 mM. Isolation of genomic DNA was continued as described in section 2.44. After

quantitation of the DNA, 6 µg aliquots of each of the DNA samples were digested with appropriate restriction enzymes. The digested DNA was run on a 1% agarose gel and transferred by the technique of Southern.

2.47 RNA Transcription Mapping

The 1.9 Kb EcoRI fragment which contains the last two exons and their flanking sequences was cloned into pGEM3 to give a plasmid known as pGMRP2. The full length MRP/PLF cDNA was subcloned into pGEM3 vectors to yield a plasmid known as pPH2GEM3 The template was linearized then high specific activity RNA probes were prepared by *in vitro* transcription using Promega Biotec T7 RNA polymerase according to the manufacturer's directions in the presence of (α-^{32}P)CTP. The reaction was allowed to proceed for 50 min then it was chased for 10 min with 125 µM unlabelled CTP to ensure the production of predominantly full length transcripts. An excess of transcripts were mixed with 10 µg of appropriate RNA in a buffer containing (80% formamide, 40 mM PIPES (pH 6.7), 0.4 M NaCl, 1 mM EDTA) and incubated at 85°C for 5 min before an overnight incubation at 50°C. The hybridization reaction was terminated by the addition of 10 volumes of RNase digestion buffer (10 mM TrisHCl (pH 7.5), 5 mM EDTA, 300 mM NaCl) containing 40 µg/ml RNase A and 4 U/ml RNase T1 (Pharmacia) and incubated at 37°C for 1 h. RNase-resistant fragments were ethanol precipitated and run on an 8% sequencing type gel.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF A MITOGEN-REGULATED PROTEIN/PROLIFERIN GENE

3.01 Abstract

A genomic clone containing the complete gene, designated mrp/plf1, of a member of the mitogen-regulated protein/proliferin (MRP/PLF) multi-gene family was isolated from a mouse recombinant library constructed in lambda Charon 4A. The DNA sequences of the promoter region, the exons, and the exon/intron boundaries have been determined. These data indicate that mrp/plf1 corresponds to a previously uncharacterized functional member of the MRP/PLF family. The size and organization of the 5 exons in the murine mrp/plf1 gene are very similar to those of members of the prolactin-growth hormone family, indicating that mrp/plf1 is a part of this superfamily. This is the first mouse gene in this superfamily to be characterized in detail.

* Some of the results presented here have been submitted for publication. Connor, A.M., Waterhouse, P., Khokha, R., and Denhardt, D.T. Characterization of a mouse mitogen-regulated protein/proliferin gene: a member of the growth hormone/prolactin gene superfamily

3.02 Introduction

The expression of eukaryotic genes involves the interaction of complex biochemical processses to transcribe, process, and transport mature mRNA before it can be translated into a protein (Leff *et al.*, 1986). Studies using a combination of *in vitro* mutagenesis and DNA-mediated gene transfer identified two types of cis-acting regulatory sequences in genes. Promoters are located close to the initiation site and act in a position dependent manner. They consist of the cap site where RNA polymerase II begins mRNA synthesis and usually the TATA box which is believed to be involved in fixing the site of initiation. Enhancers can be located far from the initiation site and act in a position and orientation independent manner. Many enhancers impose tissue-specific expression on adjacent promoters. Cis-acting elements interact with specific protein factors in order to be effective.

The coding region is usually broken up into several segments known as exons which are separated by non-coding regions of varying length known as introns. The 3' untranslated region encodes a site which specifies that a poly A tail is added to the mRNA. There is evidence that the 3' end can, in certain cases, form structures that influence the stability of the mRNA thereby affecting susceptibility of the RNA to nuclease attack (Brawerman, 1987). mRNAs of transiently expressed mammalian genes contain an AU-rich sequence in the 3' untranslated region

that appears to signal an mRNA processing pathway for the rapid degradation of these mRNAs (Shaw and Kamen, 1986).

The sequence of the mRNA and encoded MRP/PLF protein strongly suggest that it belongs in the prolactin-growth hormone family. The gene structures of the related hormones in this family have been deduced in a variety of species. All of them have 5 exons which are interrupted by 4 introns and the splice junctions have been conserved, suggesting that these genes arose from a common precursor which had similar exon/intron boundaries (Miller and Eberhardt, 1983). There have not been any published reports, however, of a mouse member of this family.

As explained in detail in chapter 1, MRP/PLF is increased in abundance in the cell culture medium of Swiss 3T3 cells after the addition of mitogens such as serum, FGF, and EGF. Our working hypothesis has been that this enhanced expression of MRP/PLF is due to increased transcription of the gene and that sequences capable of binding proteins which act as messengers for the mitogenic signal are most likely in the 5' upstream promoter/enhancer region of the gene.

As detailed in chapter 5, some change in the regulation of MRP/PLF expression occurs when non-expressing MEFs are passaged according to a 3T3 regime to become an expressing 3T3 line. Since run-on transcriptional analysis had suggested that MRP/PLF was transcribed in both MEF and 3T3 cells, we hypothesized that in this case, MRP/PLF was being

regulated at a post-transcriptional level such as RNA processing, transport or stability.

In order to address the above questions, we decided to isolate an MRP/PLF genomic clone. We initially identified its intron/exon structure in order to determine if MRP/PLF is a member of the prolactin-growth hormone family at the genetic level as well as at the protein level. We identified and sequenced the promoter region of the MRP/PLF genomic clone. We anticipated that we would be able to deduce possible mechanisms of control by comparing the sequence of this promoter with sequences of other genes which are regulated by growth factors.

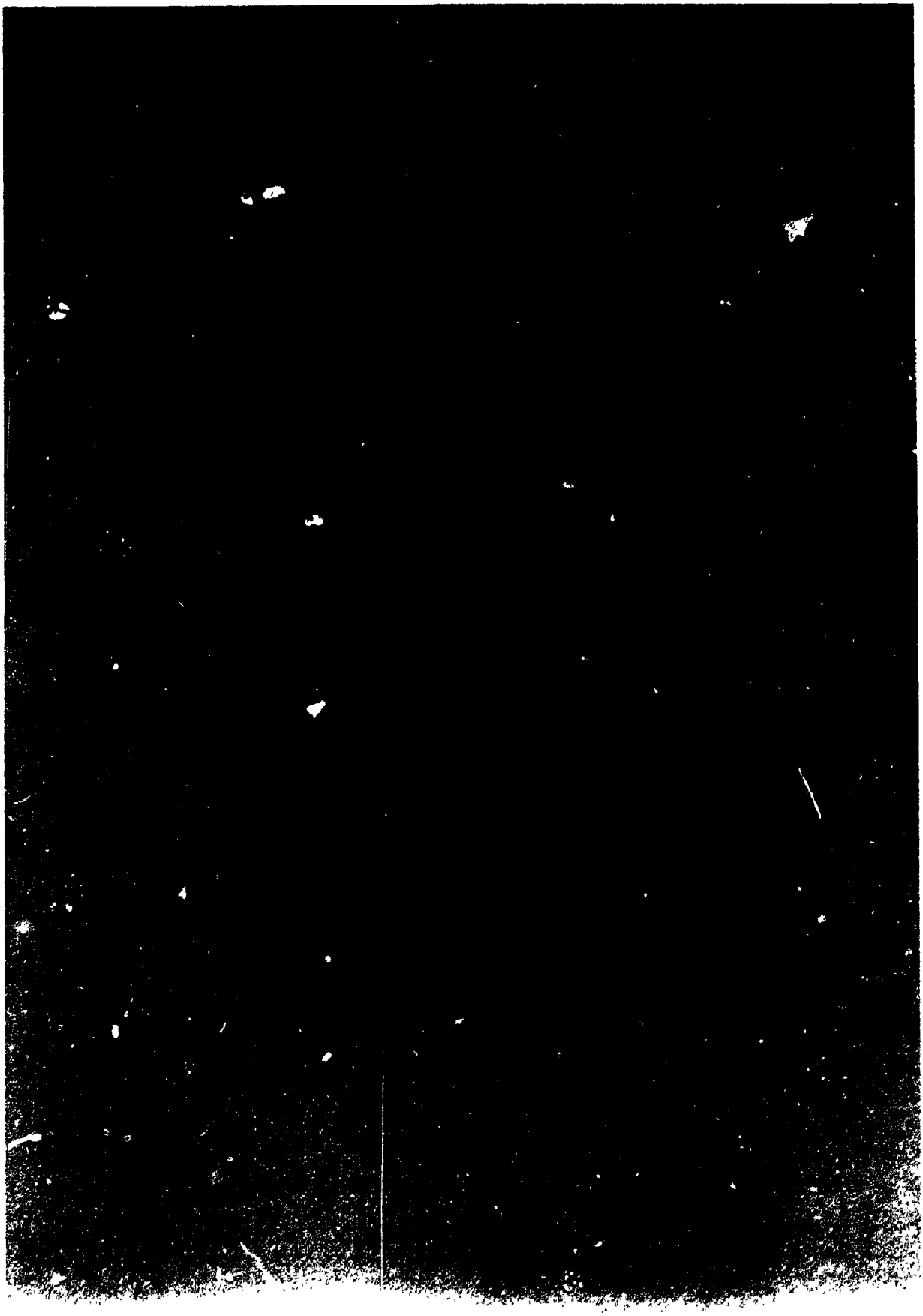
3.03 Isolation of MRP/PLF Genomic Clones from Lambda Libraries

Two different lambda libraries (section 2.06) were screened as described in section 2.13. Eleven positive clones were identified initially and were eventually purified by low density plating. Small scale lambda preparations (section 2.14) were made and cleaved with EcoRI. All 6 clones originating in the 9090 library were identical while 3 from the lambda amplified pool #4 library were identical and another 2 were different. As shown in Figure 1, 4 unique clones were isolated. The clone kept from the 9090 library is known as 6.1 while the clones from the pool #4 library are known as 1.3, 4.4, and 2.1.

Throughout the course of this work, advantage was taken

Figure 1 EcoRI Digestion of Lambda MRP/PLF Genomic Clones

An EcoRI digestion was performed on DNA isolated from the lambda MRP/PLF genomic clones which had been purified by low density plating. The digested DNA was electrophoresed on a 0.4% agarose gel and bands were visualized by ethidium bromide staining. Clone 6.1 is from the lambda 9090 library. Prior to photography, bands having molecular weights of approximately 1.9×10^4, 1.1×10^4, 8.4×10^3, 3.5×10^3 and 1.9×10^3 were observed for clone 6.1. The two largest bands are lambda arms while the others are insert fragments. Clones 2.1, 4.4, 1.3 and 3.1 were isolated from the lambda amplified pool #4 library. Although they do not show up in this reproduction, several bands were observed in each digestion because there are several EcoRI sites within the inserts. It is evident from this experiment that 1.3 and 3.1 are different isolates of the same clone, while each of the other isolated clones is unique.



of the published sequences of PLF-1 and PLF-2 (Linzer et al., 1985) to aid in orienting the gene. The sequence of PLF-2 is shown in Figure 2. Southern blots (section 2.16) of various restriction digests of the genomic clones were probed with inserts encompassing either the 5' or 3' end of the MRP/PLF cDNA. This revealed that two of the isolates, 1.3 and 4.4, lacked the 3' end of the gene but did have the 5' end. Two of the isolates, 6.1 and 2.1, appeared to represent the entire MRP/PLF gene and one of these, 6.1, was studied further. The 5' end of the gene is located on an 8.4-Kbp EcoRI fragment, the 3' end is located on a 1.9-Kbp EcoRI fragment and these two fragments are separated by a small (300 bp) EcoRI fragment. More extensive restriction maps of all the isolates were generated by single and double restriction enzyme digestions followed by Southern blotting analysis and are shown in Figure 3A. It is not possible without probing Southern blots with purified lambda arms to precisely define the boundaries for clones 1.3, 4.4, and 2.1 because of the way the MEF pool #4 library was constructed in lambda L47.1. The inserts cannot be cut out cleanly. In other words, there are junction fragments consisting of insert attached to lambda arms. It is also not known which orientation the inserts are cloned with respect to the right and left arms of lambda. Figure 3B shows that when an insert containing an internal EcoRI restriction site is cloned into the BamHI site of lambda L47.1, cleavage with EcoRI will generate 2 junction frag-

Figure 2 Sequence of PLF-2

The PLF-2 cDNA clone was isolated from a library prepared from mRNA obtained from BALB/c mouse placenta. The sequence here was taken from Linzer et al., 1985. The translational start site and termination site are indicated by *, while the poly A addition signal is underlined.

Sequence of PLF-2

```
   1  AAGGCTTCCA ACTCCAGTAA AGCATCTTCC CGGAATCCAC AGCTAAGCCT
                         *
  51  GGGTAGGACT CTGCAGAGAT GCTCCCTTCT TCGATTCAAC CATGCTCCTG
 101  GATACTGCTC CTACTACTGG TGAACAGCTC GTTATTGTGG AAGAATGTTG
 151  CCTCATTTCC CATGTGTGCA ATGAGGAATG GTCGTTGCTT TATGTCCTTT
 201  GAAGACACAT TTGAATTAGC CGGCAGTTTG TCTCATAATA TCAGTATAGA
 251  AGTTTCGGAA CTGTTCAATG AATTTGAAAA ACATTATTCT AACGTGTCTG
 301  GGCTCAGAGA CAAAAGCCCC ATGAGATGCA ATACTTCTTT CCTTCCAACT
 351  CCAGAAAACA AGGAACAAGC CAGGCTCACA CACTATGCAG CTCTTCTGAA
 401  ATCAGGAGCC ATGATTTCGG ATGCCTGGGA AAGCCCTCTG GACGATCTAG
 451  TGAGTGAATT ATCTACCATA AAAAATGTCC CTGATATAAT CATCTCCAAA
 501  GCCACAGACA TAAAGAAAAA GATCAACGCA GTCCGGAACG GGGTTAATGC
 551  CCTCATGAGC ACCATGCTTC AGAATGGAGA TGAAGAAAAG AAGAACCCTG
 601  CCTGGTTCTT GCAATCTGAC AATGAAGATG CTCGCATTCA TTCTTTATAT
 651  GGCATGATCA GCTGCCTAGA CAATGACTTT AAGAAGGTTG ATATTTATCT
 701  CAACGTCCTG AAGTGTTACA TGTTAAAAAT AGATAACTGC TGATATTTCT
 751  TTCATGTGCT CTGCTTCTGA AATATCATGT AATATCCTTT CAATTTGTAT
 801  CTTTTGAATT TGTTGTTGAC TCATTTAAAA ATAAAAAGTA GCTCTCAGAA
 851  ATATA
```

Figure 3A Restriction Maps of the Lambda MRP/PLF Genomic Clones

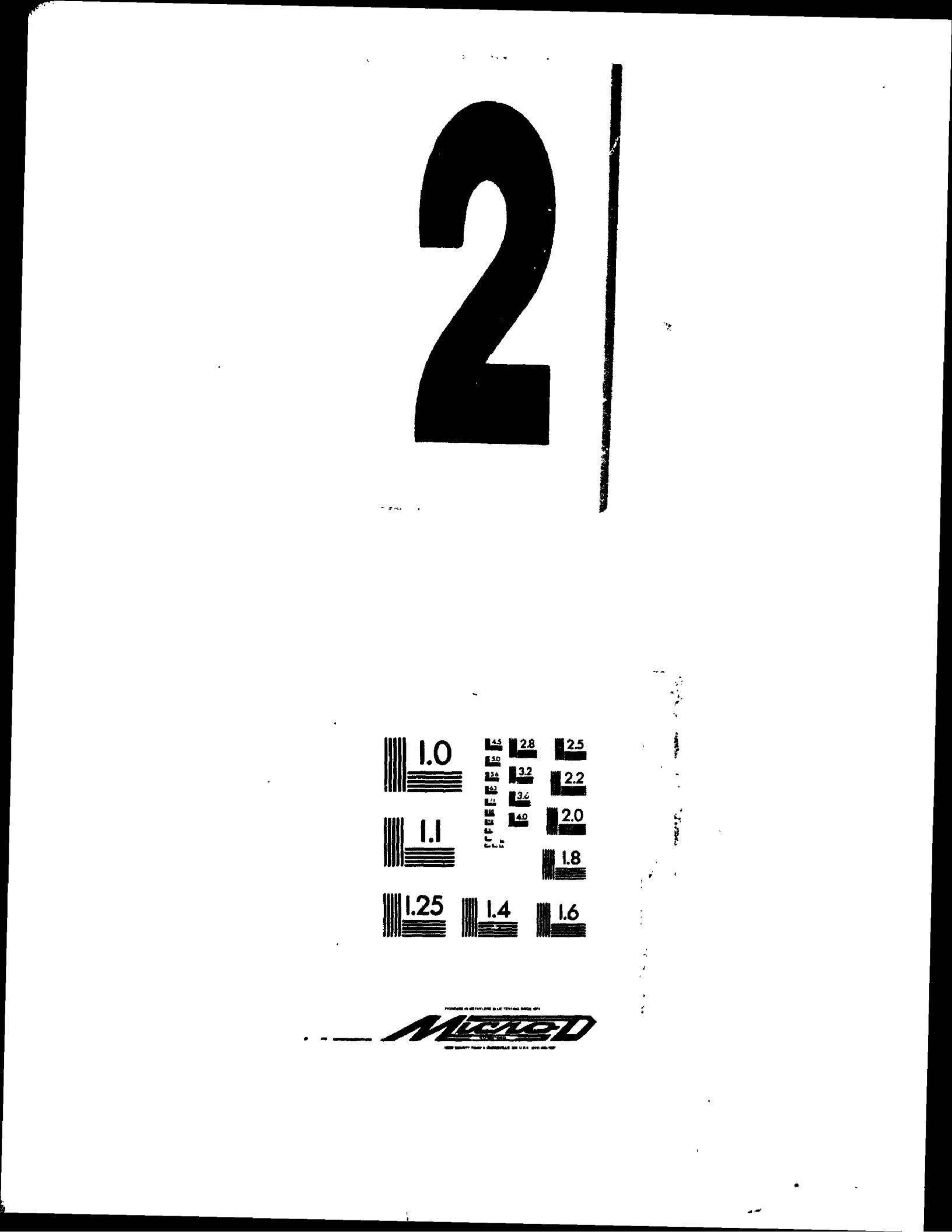
Restriction maps of the MRP/PLF genomic clones isolated from recombinant libraries constructed in Charon 4A (6.1) or lambda L47.1 (2.1, 4.4, and 3.1) are shown. The inserts are shown with the 5' end of the gene on the left. The approximately 4-Kbp region at the most 5' end of isolates 1.3 and 4.4 has not been finely mapped, hence the restriction maps shown for this area are incomplete. The fragments used to generate the subclones described in the text are shown below each isolate.

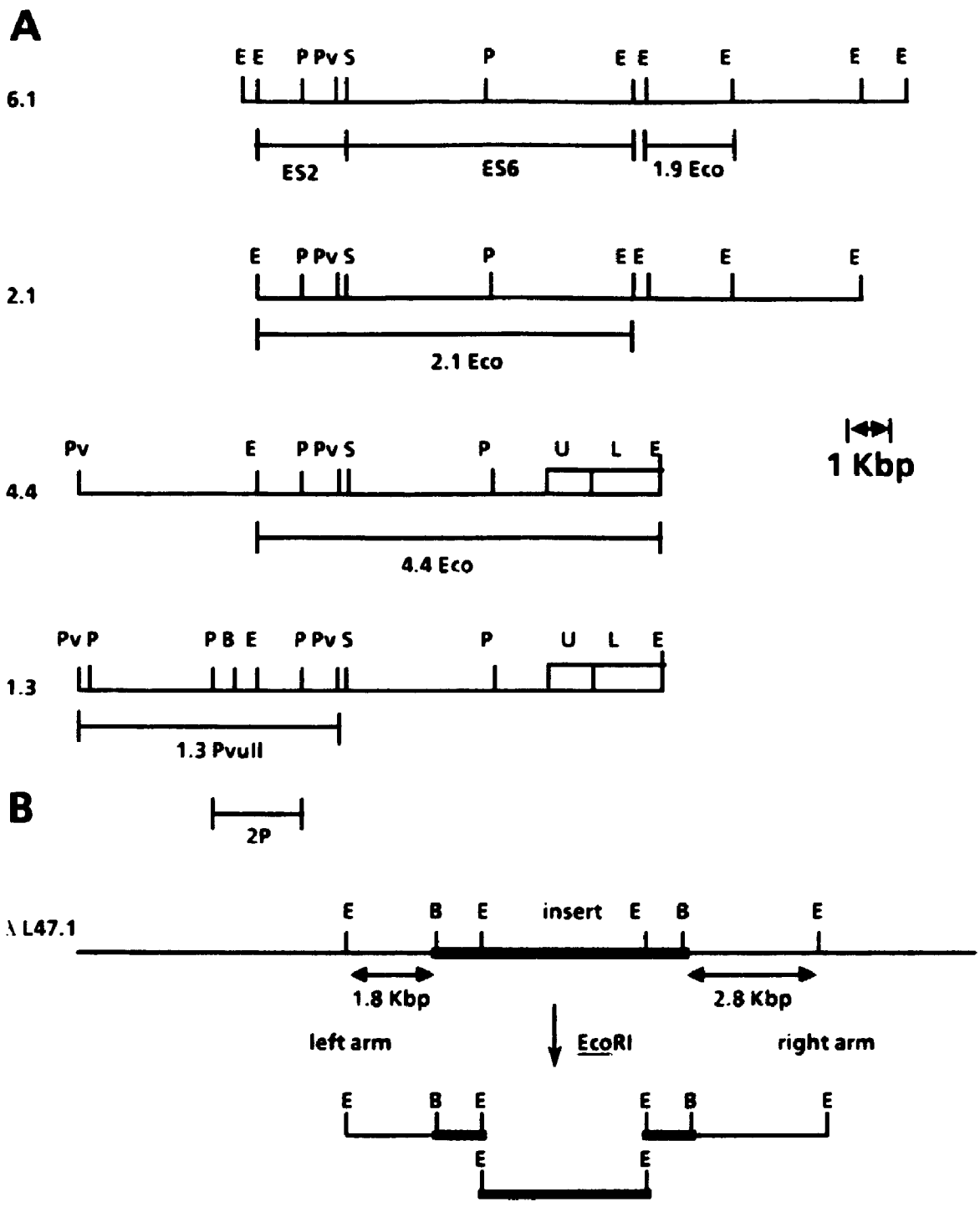
E=EcoRI, Bg=BglII, P=PstI, Pv=PvuII, S=StuI, U=unknown region corresponding to either the lambda arm or the insert, L=lambda arm

3B Cartoon of Lambda L47.1

The EcoRI cleavage of a hypothetical insert containing internal EcoRI sites, cloned into the BamHI site of lambda L47.1 is shown to illustrate the origin of the unknown region described above.

B=BamHI, E=EcoRI


2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8


A
6.1
E E P Pv S P E E E E E
ES2
ES6
1.9 Eco
2.1
E P Pv S P E E E E
2.1 Eco
4.4
Pv E P Pv S P U L E
4.4 Eco
1 Kbp
1.3
Pv P P B E P Pv S P U L E
1.3 PvuII
B
2P
λ L47.1
E B E insert E B E
1.8 Kbp
2.8 Kbp
left arm
EcoRI
right arm
E B E E B E
E E

ments. One of them will contain 1870 bp of lambda DNA from the left arm and the other will contain 2830 bp of lambda DNA from the right arm of lambda L47.1. This 1 Kbp difference in size between the two lambda arms is the region indicated on the genomic maps as unknown. If the insert was attached to the left arm at this boundary region, the unknown region would represent genomic DNA while if the insert was attached to the right arm at this boundary region, the unknown region would represent lambda DNA.

3.04 Subcloning Genomic Fragments into Plasmid Vectors

Inserts in lambda vectors are difficult to work with since the vectors and the inserts themselves are large, on the order of 60 Kbp in total. The 8.4-Kbp EcoRI and 1.9-Kbp EcoRI fragments from clone 6.1 were eluted (section 2.10). The 1.9-Kbp EcoRI fragment was subcloned directly into the EcoRI site of pBR322 to give a plasmid known as p1.9Eco. Since Southern blot analysis had revealed that the 5' end of MRP/PLF cDNA was encoded somewhere on an approximately 2-Kbp EcoRI-StuI fragment (Figure 3A), the 8.4-Kbp EcoRI fragment was digested with StuI and the two resulting fragments were subcloned into pBR322 which had been cleaved with EcoRI and PvuII. The clone representing the most 5' end of the gene is referred to as pES2, while the other clone is referred to as pES6. The approximately 9.6-Kbp EcoRI fragment of clone 4.4 and the 8.4-Kbp EcoRI fragment of clone 2.1 were subcloned into the EcoRI site of

pBR322 to give plasmids known as p4.4Eco and p2.1Eco respectively. The approximately 6-Kbp PvuII fragment of clone 1.3 was cloned into the PvuII site of pBR322 to give a plasmid known as p1.3PvuII.

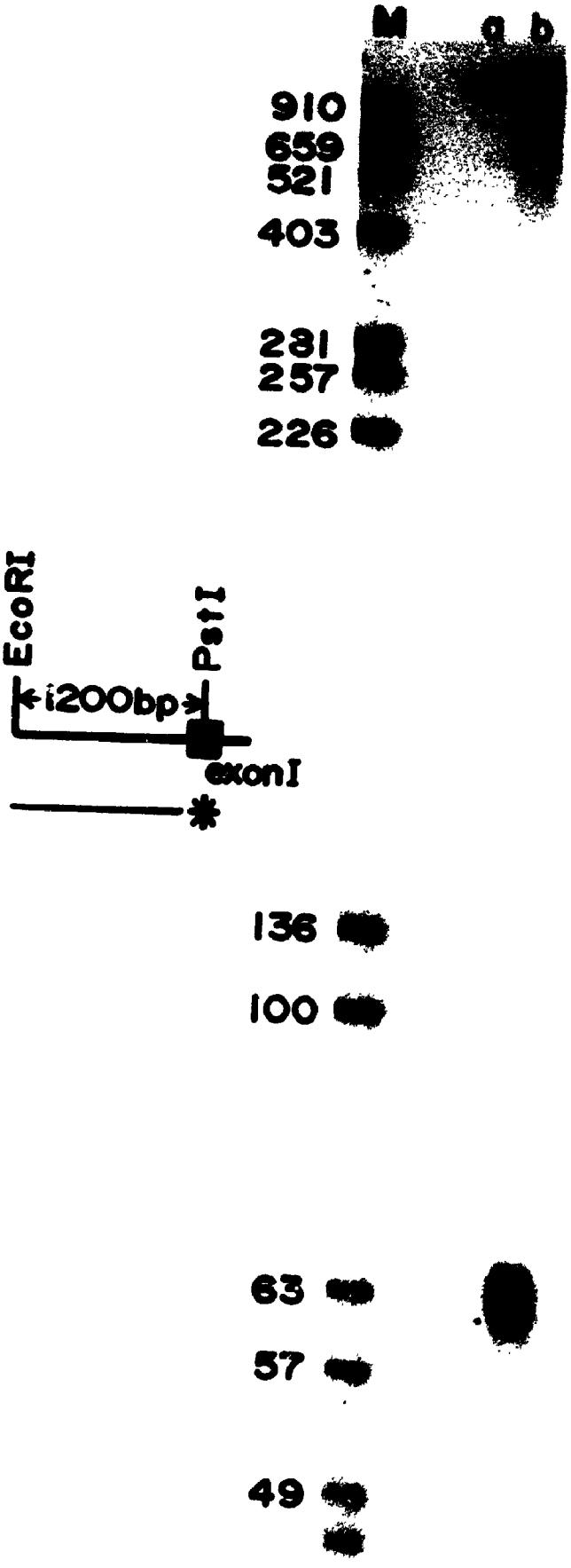
3.05 S1 Mapping the Site of Transcription Initiation

It was known from a published PLF cDNA sequence (Figure 2) that a PstI site existed just prior to the translational start site. Restriction mapping of the pES2 plasmid (section 3.04) revealed that there was a PstI site within the insert about 1.1 Kbp away from the EcoRI site. In order to locate the transcription initiation site, S1 mapping was performed as described in section 2.33. Poly (A)$^+$ RNA, isolated from BNL cells treated with TPA, was chosen as the source of RNA as we expected this would be a rich source for MRP/PLF mRNA. Growing BNL cells express MRP/PLF mRNA constitutively and the MRP/PLF levels are increased in 3T3 cells after administration of TPA (Fienup et al., 1986).

The predominant transcriptional start site was determined by comparing the position of the protected fragment with known size markers (Figure 4). Although it cannot be excluded that the heterogeneity observed at the 5' end is artifactual, it is likely that it is a consequence of variations in the actual start site either for the same gene or among the different genes in this multi-gene family because a similar hetergeneity was detected when primer extension was used to locate the transcription initiation

Figure 4 S1 Nuclease Mapping MRP/PLF RNA 5' End

Polyadenylated RNA from TPA-treated BNL cells was annealed to a 1.2-Kbp EcoRI-PstI DNA fragment containing part of exon 1 and 5' flanking mrp/plf1 sequences that was 5'-^{32}P-labelled at the PstI end. The samples were digested with S1 nuclease, denatured and electrophoresed on a 8% acrylamide-7M urea sequencing gel. Lanes:M, ^{32}P-labelled AluI-digested pBR322; a, no input RNA; b, 10 µg of polyadenylated RNA from TPA-treated BNL cells.


M
a
b
910
659
521
403
231
257
226
EcoRI
PstI
~200bp
exon I
136
100
63
57
49

site of proliferin RNA (Linzer and Mordacq, 1987).

3.06 Mapping and Sequencing the Exons

The subclones of isolate 6.1 were finely mapped using restriction enzyme digestions followed by Southern blotting using full length MRP/PLF cDNA as a probe. Convenient restriction fragments encompassing the exons were gel purified and cleaved with four base pair cutters such as AluI, DraI and HaeIII. These fragments were subcloned into M13mp18 or mp19 and plaque lifts were prepared and screened as described for lambda in section 2.13. Single-stranded DNA was prepared from plaques yielding a positive signal and was sequenced. The overall picture of the size and location of the exons is shown in the upper panel of Figure 5. It can be seen that the MRP/PLF gene contained in lambda clone 6.1 and henceforth known as mrp/plf1 is about 9.5 Kbp in size, excluding the 5' and 3' flanking regions and is made up of 5 exons which are separated by 4 introns. Table 5 shows the splicing sites follow Chambon's rule in that they have the characteristic G-T at the 5' end of the intron and A-G at the 3' end of the intron (Breathnach and Chambon, 1981). There is a polyadenylation signal (AATAAA) at the 3' end of the gene (Benoist et al., 1980).

The first mrp/plf1 exon contains 68 nucleotides of the 5'-untranslated sequences, the first 10 codons of the proposed signal peptide (Linzer and Nathans, 1984) and the first base of the eleventh codon. The second exon contains

Figure 5 **Comparison of the Gene Structure of <u>mrp/plf1</u> with rPRL and rGH**

The restriction map of the DNA locus isolated from a recombinant library constructed in Charon 4A is shown. The exons, indicated by boxes, were located by restriction mapping and analysis of Southern blots. The transcriptional start site and polyadenylation signal are located as indicated. The gene structures of rPRL and rGH have been redrawn from Cooke and Baxter (1982), and are shown here as representatives of the prolactin-growth hormone family.

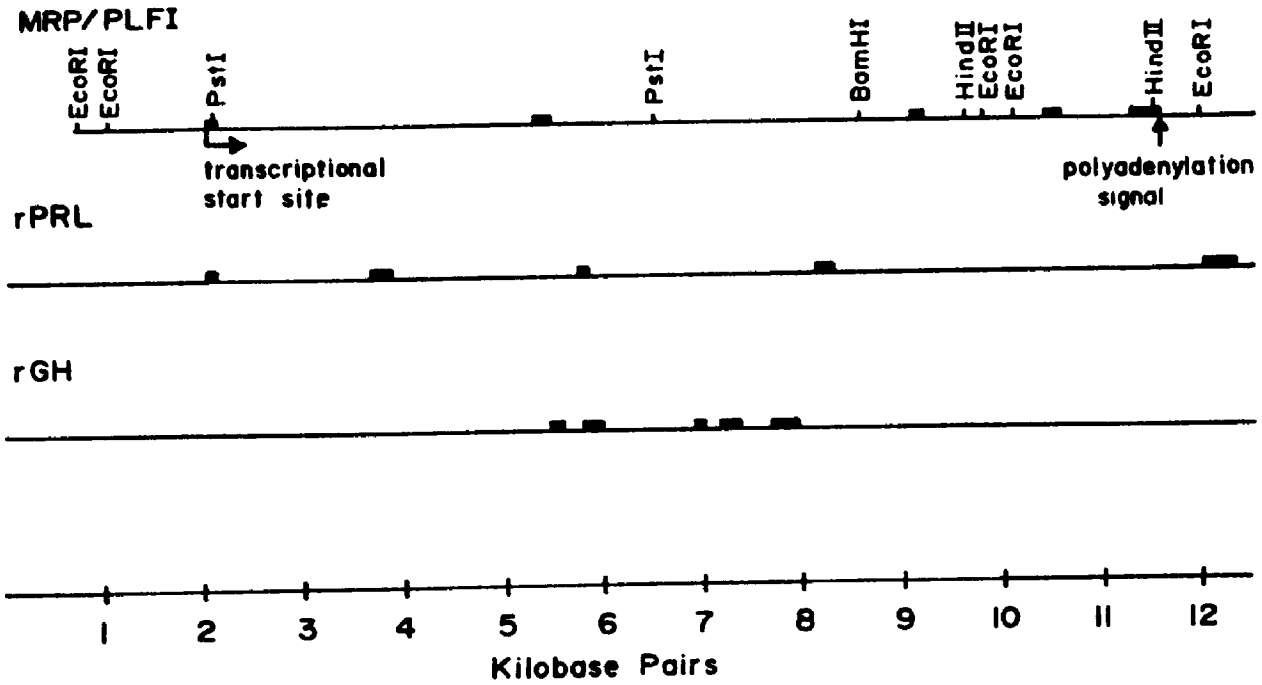

MRP/PLFI
EcoRI
EcoRI
PstI
PstI
BamHI
HindII
EcoRI
EcoRI
HindII
EcoRI
transcriptional start site
polyadenylation signal
rPRL
rGH
1
2
3
4
5
6
7
8
9
10
11
12
Kilobase Pairs

Table 5

DNA sequence of the exon-intron junctions of MRP/PLF1

cDNA position (bp)	5'cDNA border/splice donor•••/ intron•••splice acceptor/3'cDNA border
exon1 1-99	CATGCTCCT/GTAAG•••3.1-kb•••TACAG/GGATACTGC
exon2 100-275	AATGAATTT/GTAAG••••3.6-kb••••TCCAG/GAAAAACAT
exon3 276-383	CTCACACAC/GTGAG•••1.2-kb•••ATTAG/TATGCAGCT
exon4 384-566	GAGCACCATG/GTGAG•••0.7-kb•••ATTAG/CTTCAGAAT
exon5 567-855	

the remaining 2 bases of the eleventh codon, an additional 18 codons of the proposed signal sequence and the next 40 codons. The third, fourth and fifth exons contain respectively 36, 61 and 58 codons.

It was shown by Wilder and Linzer, 1986 and A.M. Craig in our laboratory that MRP/PLF is present in approximately five copies per haploid genome. Table 6 shows that the predicted mRNA product of the BALB/c mouse mrp/plf1 gene is not identical to either of the two published proliferin mRNA sequences: PLF-1, a BALB/c 3T3 cell-derived clone, or PLF-2, a BALB/c mouse placenta-derived cDNA clone (Linzer and Nathans, 1984; Linzer et al., 1985).

3.07 Comparison of mrp/plf1 with Other Members of the Prolactin-Growth Hormone Family

The lower part of Figure 5 illustrates the similarity in structure between mrp/plf1 and other genes in the mammalian growth hormone-prolactin family. The gene structures of rPRL and rGH were redrawn from Cooke and Baxter, 1982 and are shown as representatives of the growth hormone-prolactin family since there are not any published gene structures of this family available for the mouse. The rat rPRL gene is very similar in size, 10.5 Kbp compared to 9.5 Kbp for mrp/plf1, while the rGH gene is much smaller. Although the size of the exons is almost identical for members of this family, the size of the introns varies considerably. The preservation of the position of the

TABLE 6

Comparison of the Predicted mRNA Products of Different MRP/PLF Genes

Amino acid codon[a]	PLF-1[b]	PLF-2[c]	MRP/PLF1
5	TTG (Leu)	TCG (Ser)	TCG (Ser)
63	TCA (Ser)	TCG (Ser)	TCA (Ser)
67	ACT (Thr)	AAT (Asn)	AAT (Asn)
107	TCA (Ser)	GCA (Ala)	GCA (Ala)
117	TTG (Leu)	TCG (Ser)	TTG (Leu)

[a]Only codons which differ between the clones are shown.

[b]Derived from BALB/c 3T3 cells.

[c]Derived from placenta of a BALB/c mouse.

splice sites and cysteine residues in mrp/plf1 compared to rGH and rPRL shown in Figure 6, corroborates the conclusion that MRP/PLF belongs to this gene superfamily and supports the belief that all members of this family are derived from a common precursor which had similar intron/exon boundaries (Miller and Eberhardt, 1983).

It was of interest to us to determine if mouse MRP/PLF cDNA would hybridize to any identified rat members of the growth hormone-prolactin family. The blots used for these experiments were kindly provided by Dr. M. L. Duckworth, University of Manitoba. Hybridizations and washings were as described in section 2.12. A nick-translated MRP/PLF cDNA probe (1 x 10^7 cpm) did not hybridize detectably to a Northern blot of 5 µg rat placental mRNA from day 13, 15, 16, 17 or 18 of development. Similarly, signals were not observed when the MRP/PLF cDNA was hybridized to a Southern blot of 10 µg rat liver genomic DNA. However, as shown in Figure 7, when a Southern blot of various cDNA clones of the PRL-GH family was probed with MRP/PLF cDNA, the cDNA clone of rat prolactin-like protein A hybridized. As mentioned in section 1.06, rPLP-A is a cDNA clone isolated from rat placenta by virtue of its homology with the placental lactogens. Human PRL and PL as well as rPL-II, rGH, rPRL and several other rat placental clones all failed to hybridize with the MRP/PLF cDNA clone. These results indicate that MRP/PLF cDNA is more similar to the rPLP-A clone than to any other members of this family looked at,

Figure 6 Similarities in Primary Structure and Exon Organization between MRP/PLF, PRL and GH

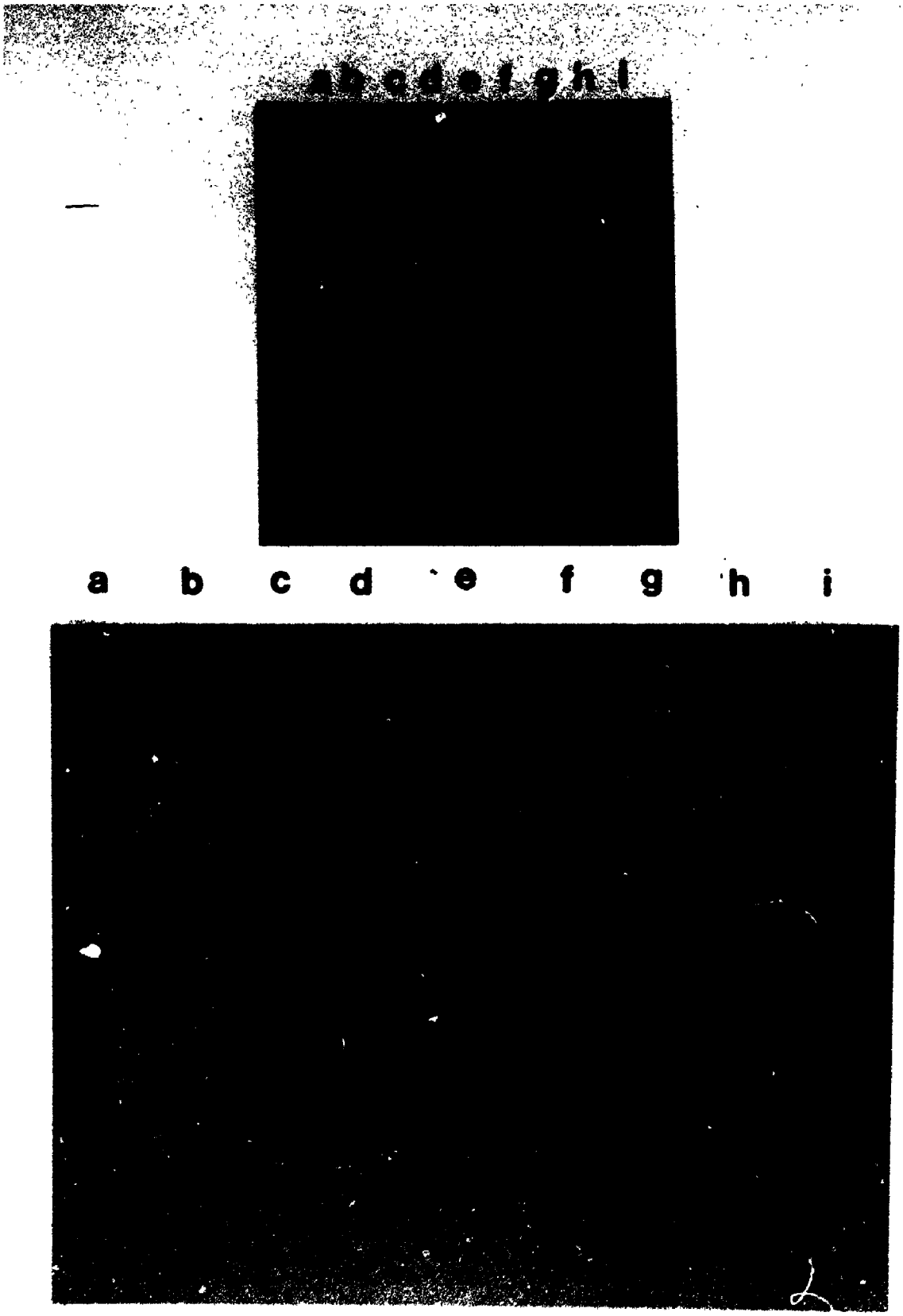
The predicted amino acid sequence of mrp/plf1 is aligned with the rPRL and rGH sequences to show the similarity of the splice site locations in this family. The rPRL and rGH sequences were aligned as in Cooke and Baxter (1982), and mrp/plf1 was aligned by inspection. Asterisks indicate identical amino acids in mrp/plf1 and rPRL.

```
EXON 1
MRP/PLF1    MLPSSIQPCS
            *  *
rPRL        MN-SQVSARK
rGH         M-----AA-D

EXON 2
MRP/PLF1       WILLLLLVNSSLLWK-NVASFPMCAMRNGRCFMSFEDTFELAGSLSHNISIEVSELFNEF
                  ****  * **   **   * *    * *       *     *** *       * **
rPRL           AGTLLLLMMSNLLFCQNVQTLPVCS--GGDCQTPLPELFDRVVMLSHYIHTLYTDMFIEF
rGH         SQT-PWLLTFSLLCLLWPQEAGALPA---------MPLSSLFANAVLRAQHLHQLAADTYKEF

EXON 3
MRP/PLF1    EKHYSNVSGLRDKSPMR-----CNTSFLPTPENKEQARLTH
             * *        *         * ** * *** ****
rPRL        DKQYVQDREFIAKAIND-----CPTSSLATPEDKEQAQKVP
rGH         QRAYIPEGQR--YSIQNAQAAFCFSETIPAPTGKEEAQQRT

EXON 4
MRP/PLF1    YAALLKSGAMILDAWESPLDDLVSELSTIKNVPDIIISKATDIKKKINAVRNGVNALMSTM
               **         *  **  *   *  *   ** *** *  *         *     *
rPRL        PEVLLNLILSLVHSWNDPLFQLITGLGGIHEAPDAIISRAKEIEEQNKRLLEGIEKIISQ
rGH         DMELLRFSLLLIQSWLGPVQFLSRIFTNSL-----MFGTSDRVYEKLKDLEEGIQALMQ

EXON 5
MRP/PLF1      LQNGDEEKKNPAWF----LQSDNEDARIHSLYGMISCLDNDFKKVDIYLNVLKCYMLKIDNC
                          *     **   *       *  * **  *  *** **  * *      **
rPRL         AYPEAKGNEIYLVWSQLPSLQGVDEESKDLAFYNNIRCLRRDSHKVDNYLKFLRCQIVHKNNC
rGH         ELEDGSPRIGQILKQTYDKFDANMRSDDALLKNYGLLSCFKKDLHKAETYLRVMKCRRFAESSCAF
```

Figure 7 Hybridization of an MRP/PLF cDNA Clone with Other Members of the PRL-GH Family

A Southern blot with various members of the PRL-GH family in the form of cDNA clones, was kindly provided by Dr. M. L. Duckworth, University of Manitoba. A picture of the ethidium bromide-stained gel before transfer is shown in the upper part of this figure. Lanes: a, human placental lactogen; b, human prolactin; c, rat placental clone 54; d, rat placental clone 9; e, rat prolactin-like protein A; f, 5' end of rat placental lactogen II; g, rat placental lactogen II without the 5' end; h, rat prolactin i, rat growth hormone

The lower part of this figure is an autoradiogram of the blot probed with nick-translated full length MRP/PLF cDNA insert at 42°C. Exposure was for 5 days.


a b c d e f g h i

but since hybridization was not observed with either rat placental RNA or rat liver genomic DNA their degree of similarity is not particularly high. The fact that MRP/PLF is expressed specifically during mid-gestation while rPLP-A is expressed during late gestation suggests that they are unlikely to be functional equivalents of each other. The failure to detect a signal in either rat pla- cental RNA or rat liver genomic DNA implies that there probably is not a rat gene version of MRP/PLF. This does not mean that there might not be rat genes whose protein product has the same function as MRP/PLF.

3.08 A Poly(dT-dG).Poly(dC-dA) Sequence is in mrp/plf1

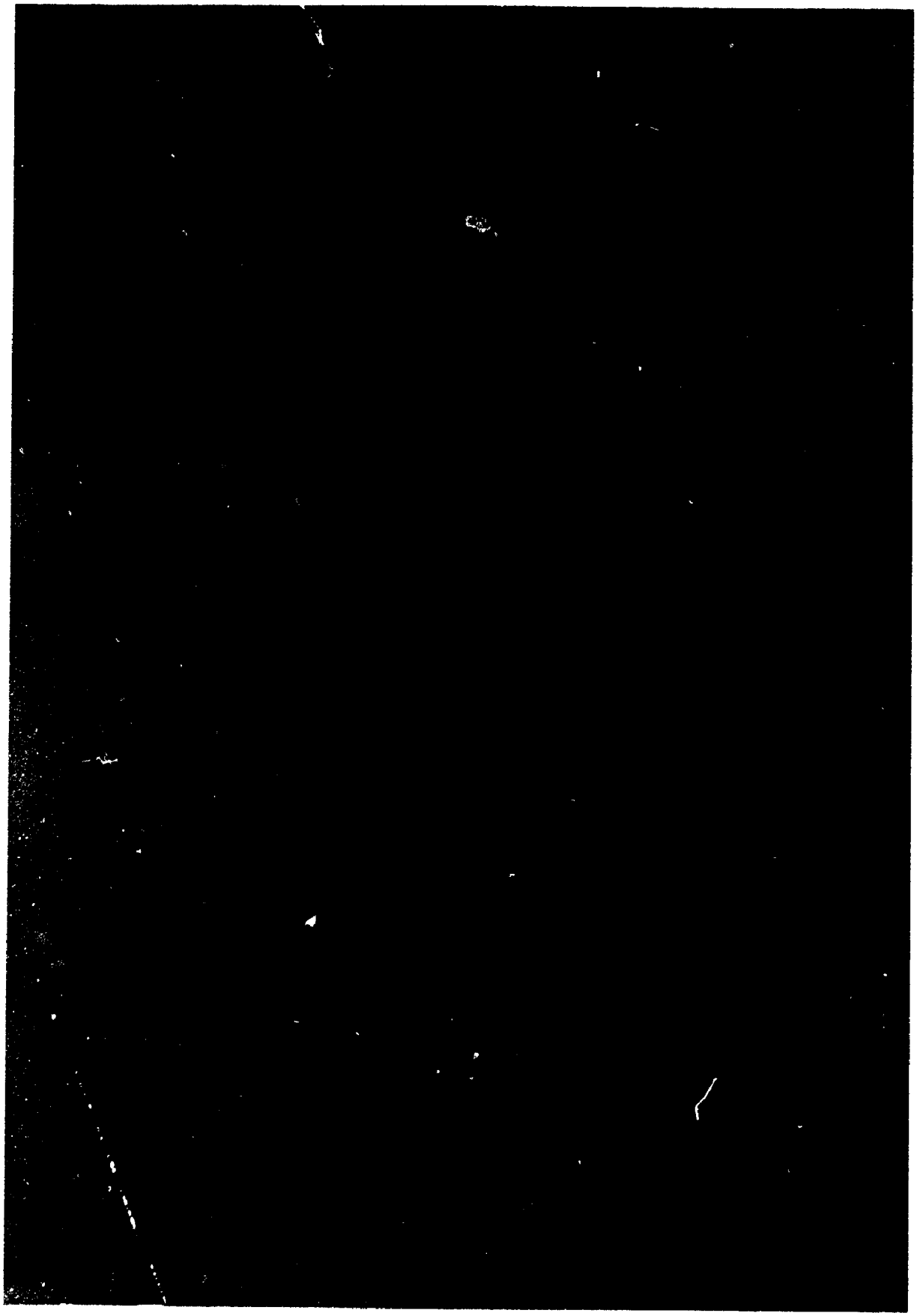
While sequencing within the vicinity of exon 3, a poly(dT-dG).poly(dC-dA), (TG-element) was found in a 1-Kbp BamHI-HindII genomic fragment (see Figure 5). This element consists of 32 repeats of (dT-dG).(dC-dA), 26 of which are uninterrupted. As shown in Figure 8, this element is not flanked by direct or inverted repeats although there is a pair of direct repeats on the 5' side indicated by the boxes.

The features of this TG-element are typical of other characterized TG-elements. They generally consist of 20 to 60 base pairs of poly(dT-dG).(dC-dA), they do not seem to have arisen by insertion or be associated with other repet- itive elements, and they are often found in introns or near coding sequences (Hamada et al., 1984). Under certain

Figure 8 A Poly(dT-dG).Poly(dC-dA) Sequence in mrp/plf1

A: The region of the sequencing gel encompassing the poly(dT-dG).poly(dC-dA) sequence found in the vicinity of the mrp/plf1 third exon is shown.

B: The sequence of the poly(dT-dG).(dC-dA) sequence deduced from the gel shown above is presented. The boxed areas indicate a pair of direct repeats.



conditions, they are able to adopt a Z-DNA conformation.

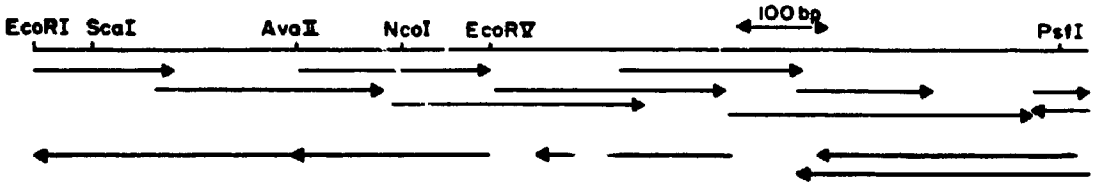
Genes which have been reported to have TG-elements include 2 members of the human actin gene family (Hamada et al., 1984), the rat prolactin gene which contains at least six of these repeats (McFarlane et al., 1986) and two human gamma globin genes (Slightom et al., 1980). In the latter case, the TG-element is located in the same intron in both genes and it has been proposed that the element might serve as a hot spot for gene conversion. This would be an interesting hypothesis to account for the MRP/PLF gene family similarity to each other. Proof of this will have to wait until other MRP/PLF genes are characterized to see if they contain TG-elements in a similar location. It should be emphasized that this TG-element was found in mrp/plf1 by chance and there may be other such elements within the gene. As the entire structure of the gene is now known, it would be relatively simple to screen it by generating known restriction fragments and hybridizing them to an oligo (dC.dA) probe.

3.09 Sequence of the 5' End of mrp/plf1

The strategy used to sequence the region of mrp/plf1 from the EcoRI site shown mapping at 1 Kbp in Figure 5 to the end of the first exon is illustrated in the top panel of Figure 9. The sequence of this region is presented in the lower panel of Figure 9. There is a TATA box located between 23 and 29 bases upstream of the transcriptional

Figure 9 Sequence of the 5' Regulatory Region of mrp/plf1

The upper portion of the figure details the strategy used to determine the DNA sequence of the 1.2-Kbp EcoRI-PstI segment of mrp/plf1. The corresponding overlapping fragments were sequenced by the dideoxy chain termination method. Almost 90% of the region was sequenced on both strands and every region was sequenced at least twice. The lower portion of the figure shows the nucleotide sequence for mrp/plf1 determined from the EcoRI site to the end of the first exon. Transcription starts at the A designated +1 that is 22 nucleotides downstream of a good TATA box (see Figure 4). Numbers on the left refer to the first base on each line.



Sequence of 5' Upstream Region of mrp/plf1

-1101
CAGGCATGGT TGGAAAATGT AGCAAGTAAA ACCACAACGT TTCTGAATAA TCCCAAAAAC AGAGTACTTA
-1031
GCTTACTAGG AGCTATTCAA CAGTAAGCCT TGGTGTCCAT CTATGTAAAA CAAAGTATTG TTAGAGAAGC
-961
CTATTTAAAT ACTAGAGATA CAGTAAAATA CCGTATGGTT TGGTTGCATG ATAAGAATAA ATTGGACTTG
-891
TAGTGAGGTA GAAGGCCACA GAGTTTTGAC TTACTCTGAT AGTACCCATG TCACACTGCT CTGTCTGTAC
-821
TAGTAGGAAA TGTCAATAAA GGACCTATTC AGCACAAGAT AACTTAACCA CCAAGGTGAA GCATGTTTTG
-751
ACTTGTTCAC TTTTAATATG ACTGTTATCA TTGAAATCAA ATATGTTCTA TTAGCTTTAA AGCCAGTTCC
-681
ATGGACTTAG GTGATGTTTG TAAGCTTGCC TGAGTATCTG TGACAGGAGA TGTTCTTGGC TACAAAAATG
-611
GAAGCTTTTG ATAATTTTTG GTTAAGTTAA TATGATATCT TTGTTAAAAT AGCCTTCAAG ACATCTGTAT
-541
TTGAATGTTC AATTGTCACT CTTATGTGCC TAATATGAAA AATCAAGCAA CTCACAATGT TCCTTGGGTG
-471
TTGTCAAAGA CTCATGAGGA TAAAACTCAA TATGGGTGAC TTTTACTGAG GCTTTGAGTC CCAACGGTCT
-401
AAAGACGATG AAATATATAT GACCTTCATC TACGTGTCAT TGAAAATTTT AGAATATATA AAATCCTGAA
-331
GGTAAAAAGG TCTATGGCAT AGCTATTTAT TTAAAGCAAG TAATTTCTAT CAGTCTTTAT GAGGAAGACA
-261
TAGTTGTGGC TACTCACAGT ATGATTTGTT TTAGTCAGAG CATGAACATG AGATGAATCA CATACATAGA
-191
ACTGACTATA TCAGTGAATC TAAAAAATAA GATAAACCAT GAACTTAGAA CTATATTAGT GTTCATGGTA
-121
TCTAGGCTAC TTAACCCTGA GGGAAAAATA ATGGAAATTA ACACGTCCTT TTCTTCCTGC CACTGTGTAA
-51 +1
CCTCAGGGTT ACTGGGATAG ACTATATAAG AGGTAGAAAA TAAGGACATA GAAGGGTTCC AACTCCAGTA
+20
AAGCATCTTC CCGGAATCCA CAGCTAAGCC TGGGTAGGAC TCTGCAGAGA TGCTCCCTTC TTCGATTCAA
+90 M etLeuProSe rSerIleGln
CCATGCTCCT
ProCysSer

start site which was identified by S1 mapping (section 3.05). Sequencing of as many as 1100 bases further upstream has failed to reveal a concensus sequence for a "CAAT box". The other members of the prolactin-growth hormone family also lack a consensus "CAAT box" (Miller and Eberhardt, 1983). The "CAAT box" was originally proposed to be analogous to the -35 region of prokaryotic promoters. A computer signal search analysis of 168 eukaryotic POL II promoters questions this proposal and suggests that some of the "CAAT boxes" identified in the literature may be there just due to statistical probability and may not have a defined function (Bucher and Trifonov, 1986).

There are a number of interesting features present in the upstream region of mrp/plf1. The locations of these elements and a brief description of their significant features are summarized in Table 7.

The direct repeats (-869 to -860, and -757 to -748) may serve as binding sites for a regulatory protein or they may indicate that the encompassed region was inserted into the genome by a mechanism resulting in the duplication of the host target DNA at the integration site.

The 11 bp inverted repeat (-110 to -100 and -51 to -41) is immediately upstream of the TATA box. This is approximately the same location as an inverted repeat found in the human GH and human PL promoters (Miller and Eberhardt, 1983), suggesting the conservation of a site for protein interaction. A region of dyad symmetry has also been

Table 7

Potentially Important Sites in the *grp/plf1* Promoter

Position	Sequence	Significant Features[a]
-869 to -860	GTTTTGACTT	a 10-bp direct repeat
-757 to -748	" "	" "
-110 to -100	TAACCCTGAGG	an 11-bp inverted repeat
-51 to -41	" "	" "
-708 to -703	TGTTCT	a half-site glucocorti-
-631 to -626	" "	coid response element (GGTACANNNTGTTCT)
-543 to -536	ATTTGAAT	7/8 bp match of the oct- amer binding protein site (ATTTGCAT)
-91 to -84	ATGGAAAT	7/8 bp match to the oct- amer binding protein site (encoded on the opposite strand)
-231 to -225	TTAGTCA	AP1 consensus binding site (TT/GAGTCA)
-208 to -202	TGAATCA	6/7 bp match to the AP1 consensus binding site

Table 7 cont'd.

Position	Sequence	Significant Features
-101 to -96	GGGAAA	serum response element of heat shock protein 70
37 to 45	CCACAGCTA	8/9 bp match to the AP4 binding site (CAGCTGTGG); encoded on the opposite strand
47 to 55	GCCTGGGT	7/8 bp match to the AP2 binding site (GCCTGGGG)
-231 to -225	TTAGTCA	6/7 bp match to the cAMP responsive element (T/G)(T/A)CGTCA
-763 to -755	AAGCATGTT	7/9 bp match to the Sph
-660 to -652	AAGCTTGCC	motif AAG(T/C)ATGCA
-298 to -290	AAGCAAGTA	" "
-224 to -216	GAGCATGAA	" "
-157 to -149	AACCATGAA	" "
-817 to -806	AGGAAATGTCAA	similar to element 1 in the E1A flanking region AGGAAAGTGA(C/A)A(A/T)

[a] sequences from Jones et al., 1988 and Wu et al., 1987

observed in other eukaryotic promoters such as human insulin and mouse β-globin.

Unlike growth factors which, as explained in section 1.01, act by binding to transmembrane receptors thereby generating a series of early responses which somehow transduce the signal to the nucleus, steroid hormones and TPA enter cells and bind to specific intracellular receptors which then interact with other proteins or specific DNA sequences (Yamamoto, 1985). Recent experiments using band-shift assays, have shown that the glucocorticoid receptor element has dyad symmetry and the two halves are not equivalent. The receptor initially binds to the TGTTCT half site and then a second molecule binds to the TGTACA half site in a co-operative manner (Tsai et al., 1988). As shown in Table 7, there are 2 of the TGTTCT half sites in the mrp/plf1 promoter. Sequences resembling the second half site can be found in the vicinity of these half sites although the spacing is more than allowed in the consensus sequence.

An AP1 binding site is the site to which AP1, a trans-acting factor whose activity is regulated by TPA, binds. Its purpose may be to transmit the growth response signals formed by the activation of PKC to the elements involved in transcription. It is interesting that the region from -231 to -225 is not only a consensus AP1 binding site but also has a 6/7 bp match to the cAMP responsive element. This would suggest that induction by TPA and cAMP is transmitted

through the same DNA binding protein. This was previously suggested by workers looking at the regulation of the proenkephalin gene by cAMP and TPA (Comb et al., 1986). The cAMP responsive element is found in a number of promoters that are induced by increasing cAMP levels such as the somatostatin and E2A genes (Jones et al., 1988).

Many of the other potentially important sites mentioned in Table 7 were originally defined by workers studying the SV40 enhancer. The octamer binding protein site is found in the SV40 enhancer and immunoglobulin promoter and enhancer elements. AP2 is a trans-acting factor induced by TPA and cAMP. AP2 and AP4 binding sites are both found in the SV40 core enhancer. The Sph motif is found as a direct repeat in the SV40 enhancer while element 1 in the E1A flanking region appears to be essential for E1A expression. The serum response element of heat shock protein 70 is that region of the DNA to which the serum response factor binds thereby conferring responsiveness to serum on the gene (Wu et al., 1987). This is not the same serum response element that has been defined in the c-fos gene suggesting that there are at least two mechanisms that enable genes to respond to serum.

It appears as if enhancers are made up of a number of short sequence motifs such as the ones just described (Ondek et al., 1988). These sequence motifs bind trans-acting nuclear factors which have little enhancing activity by themselves but act synergistically to give high levels

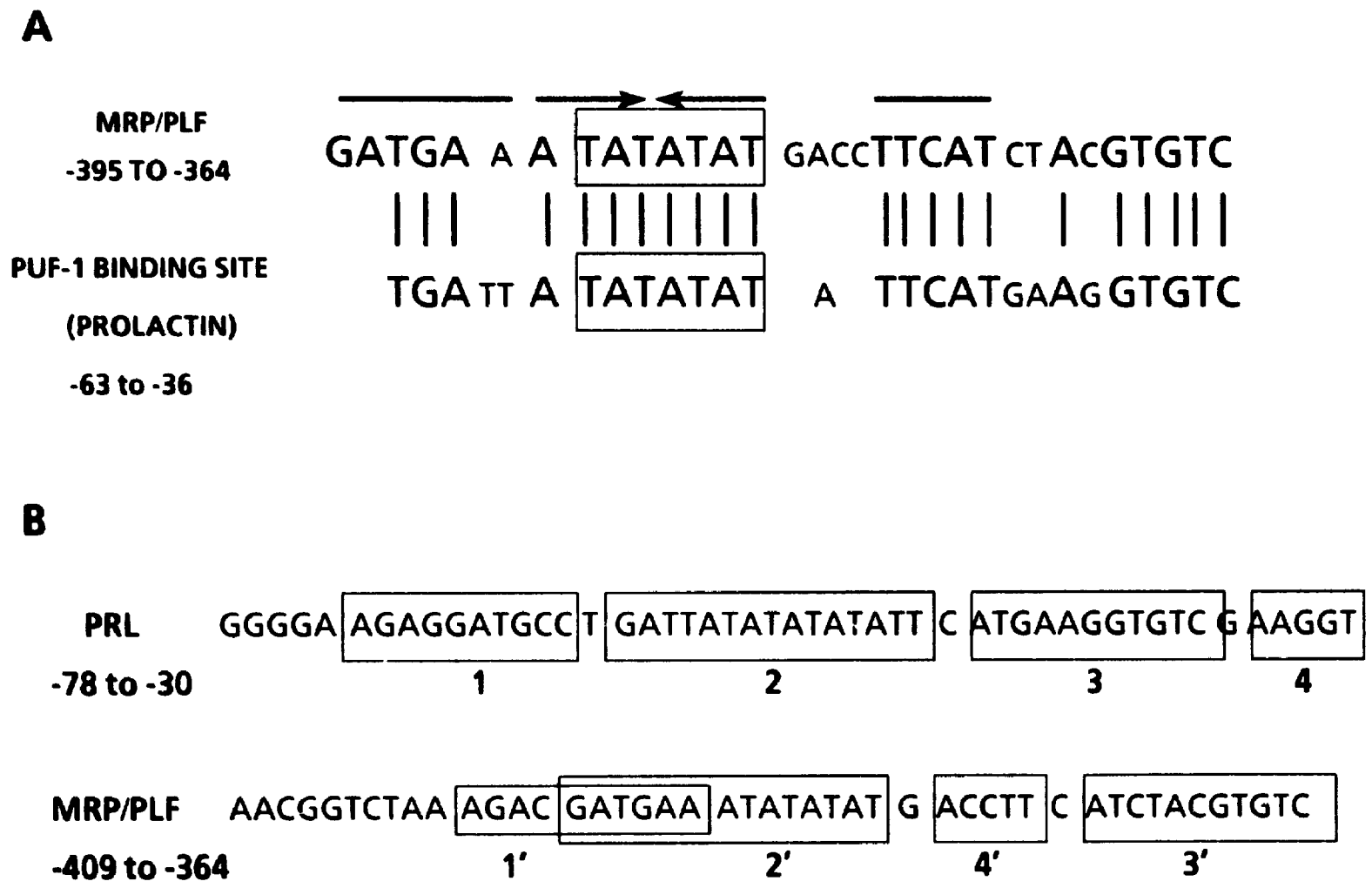
of enhancing activity. It is therefore interesting to note that in the mrp/plf1 promoter, the 10-bp direct repeat at position -757 to -748 overlaps an Sph1 motif at -763 to-755, the 11-bp inverted repeat at -110 to -100 overlaps the serum response element at -101 to -96, the AP1 consensus binding site and cAMP responsive elements located at -231 to -225 are next to a Sph motif at -224 to -216 and the AP4 binding site at 37 to 45 is close to the AP2 binding site at 47 to 55.

The region from -395 to -364 seems to be a particularly interesting region. As shown in Figure 10A, it is very similar to the prolactin upstream factor 1 (PUF-1) binding site. PUF-1 is a cell-specific transcription factor, isolated from GH3 cells which constitutively produce high levels of PRL (Barron et al., 1989). This group, who were looking at the PRL promoter, suggested that the binding of PUF-1 prevented binding of the initiation complex to the TATA box located in this element. The S1 mapping described in section 3.05 did not reveal transcripts initiating at this second TATA box in the mrp/plf1 promoter, but if it is only a weak promoter, they might not have been observed unless the gel was exposed to film for a long period of time. Alternatively, there might have been a factor bound to this region which interfered with the use of this second TATA box. It is interesting to note that the RNA was isolated from BNL cells that had been treated with TPA. Another group has used foot-print analysis to map the

Figure 10 A Sequence in mrp/plf1 Similar to that in a PRL Regulatory Region

A: The region from -395 to -364 in the mrp/plf1 promoter is shown aligned with the PUF-1 binding site, Barron et al., 1989, at position -63 to -36 in prolactin. An imperfect palindrome is indicated by arrows and the TATA box element is enclosed.

B: The region in the mrp/plf1 promoter from position -409 to -364 is compared with similar sequences in the mouse prolactin gene from -78 to -30 which have been shown by DNase footprinting to bind factors regulated by TPA and EGF (Elsholtz et al., 1986). Box 1' has a 7/10 bp similarity to box 1; box 2' has a 12/15 bp similarity to box 2; box 3' has a 8/11 bp similarity to box 3; box 4' is the same as box 4 except that it is encoded on the opposite strand.


A
MRP/PLF
-395 TO -364
GATGA A A TATATAT GACCTTCAT CT ACGTGTC
PUF-1 BINDING SITE
(PROLACTIN)
-63 to -36
TGA TT A TATATAT A TTCATGAAG GTGTC
B
PRL
-78 to -30
GGGGA AGAGGATGCC T GATTATATATATATT C ATGAAGGTGTC G AAGGT
1
2
3
4
MRP/PLF
-409 to -364
AACGGTCTAA AGAC GATGAA ATATATAT G ACCTT C ATCTACGTGTC
1'
2'
4'
3'

sequences in the PRL gene which are responsive to TPA and EGF. As shown in the upper panel of Figure 10B, they defined the region of dyad symmetry as well as about 15 bp on either side as regions of trans-acting factor binding (Elsholtz et al., 1986). While it is not known how many trans-acting factors were responsible for the footprint observed, similar regions exist in the mrp/plf1 promoter and are shown in the lower panel of Figure 10B. If MRP/PLF is regulated in an analogous manner, which is quite likely since it is in the same family as PRL, then it is possible that a factor present in response to TPA was bound to the region of dyad symmetry and prevented initiation of transcription from the second TATA box.

Good matches were not found in the mrp/plf1 promoter for the interferon consensus sequence (Friedman and Stark), or the serum response element in c-fos (Treisman, 1985).

3.10 Comparison of the 5' Sequence of mrp/plf1 to the Same Region of Two Proliferin Genes

After this work was initiated, a paper was published in which the 5' end (-578 to the end of the first exon at +99) of two different proliferin genes, PLF42 and PLF149, was examined (Linzer and Mordacq, 1987). These two promoter regions are 97% similar to each other. At the nucleotide level, mrp/plf1 is 98.4% similar to PLF42 and 97.4% similar to PLF149. Table 8 outlines the differences of these 3 promoter regions. The change at position -204 affects the

Table 8

Comparison of Nucleotide Residues of Different MRP/PLF Promoters

Nucleotide Residue[a]	PLF42	PLF149	MRP/PLF1
-532	A	C	C
-416	A	A	G
-371	C	T	T
-302	T	C	T
-204	T	C	T
-200	A	G	A
-181	T	A	T
-177	C	T	T
-176	A	G	G
-173	C	C	T
-129	G	T	T
-94	A	T	A
-93	T	A	T
-91	A	C	A
-90	T	A	T
-85	A	-	A
-59	G	G	C
-34	T	A	T
5	C	C	G
19	G	G	A
23	C	A	C
29	C	T	C
31	C	T	C
82	T	T	C

a) Only nucleotides which differ between the promoters are shown.

potential AP1 binding site in PLF149 while the changes at residues -91, -90 and -85 affect a potential octamer binding protein site in PLF149. The differences observed in the PLF42 and *mrp/plf1* promoters do not affect any of the sites mentioned in Table 7. It should be noted that PLF42 and PLF149 both match the PLF-I cDNA sequence in the region encompassing the first exon. The significance of this work is that the entire gene was isolated as a single genomic clone. For example, proliferin-related protein (section 1.06) is 37% similar to MRP/PLF at the amino acid level and is 95% similar to MRP/PLF at the nucleotide level in the first exon. The region of homology encompasses at least 97 bp and it is likely that this homology extends into the 5'-flanking region (Linzer and Nathans, 1985). Therefore unless another exon is looked at in addition to exon 1, one cannot be sure whether the 5' end represents that of an MRP/PLF gene or a proliferin-related protein gene.

3.11 5' End Sequence of the Other Genomic Clones

As reported in section 3.03, four different MRP/PLF genomic clones were initially identified and one, known as 6.1/*mrp/plf1* was studied in detail as outlined in this chapter. In order to determine whether the four isolates represented the same or different genes, the 1.2-Kbp *Eco*RI-*Pst*I piece, which represents part of the first exon and 5' flanking sequences, was gel purified from each of the iso-

lates and subcloned into the EcoRI and PstI sites of pSP65. This resulted in clones referred to as p4.4PE, p1.3PE, and p2.1PE which are the EcoRI-PstI upstream fragment of lambda clones 4.4, 1.3 and 2.1 respectively, cloned into pSP65. Each of these clones was digested with EcoRI and subjected to Bal-31 exonuclease as described in section 2.18. Deleted inserts were released from the vector by a PstI cleavage and were subcloned into the SmaI-PstI sites of M13mp19 for sequencing. The entire 1.2-Kbp regulatory region of 4.4 and most of 2.1 was sequenced while only about 300 bp immediately upstream of the PstI site of 1.3 was sequenced. In all cases, the sequence was found to be identical to that obtained for lambda clone 6.1.

3.12 Discussion

Four different MRP/PLF genomic clones were isolated from two different libraries. One of the libraries was constructed from DNA isolated from BALB/c mouse embryos while the other library was constructed from DNA isolated from Swiss mouse embryo fibroblasts. When the sequence of the 5'-flanking regions of these clones was determined, it was found to be the same in all cases. Although the MRP/PLF genes are highly conserved (see Table 8), they do show some base changes. These could be due to sequencing errors although it is unlikely for mrp/plf1 since the same region was sequenced in 3 independently isolated clones and found to be the same in all cases. The sequencing data

obtained in this study strongly suggest that the same gene was isolated in the form of 4 different clones. It is possible of course that if a larger region had been sequenced, differences would be found. Considering there are at least 5 copies of the mrp/plf gene in the mouse genome, and that 3 different 5' regions have been described (Table 8), this result is unexpected. It cannot be explained by mouse strain variation since the same gene was isolated from both BALB/c and Swiss mice.

One possible explanation arises if one assumes that the restriction enzyme used in constructing the library cut the genes in such a manner that the resulting fragments were either too large or too small to be cloned into the lambda vector. This explanation can be ruled out since one of the libraries was constructed from DNA which had been digested with Sau3A while the other one was from DNA which had been digested with BamHI, BglII, BclI, or MboI. Reverse transcriptase is often responsible for errors encountered in cDNA libraries. However since it was not used in the construction of these genomic libraries, it cannot be responsible for the results obtained here.

It can be asked whether this gene is functional. The only way of truly answering this would be to isolate the corresponding cDNA. As shown in Table 6, all of the cDNAs are very similar so it would not be easy to screen specifically for one of them. An alternative would be to isolate many MRP/PLF cDNAs and sequence them all. If the one in

question does not appear in such a screen it is still uncertain as to whether it is not there because it is not expressed at all or whether it is expressed in such low levels that it is difficult to detect. Wilder and Linzer, 1986, used oligonucleotide probes that included the sequence differences at codons 63 and 67 to screen Northern blots of RNA obtained from various cell lines and placenta. While they were able to detect sequences hybridizing to the PLF-2 probe, they were unable to pick up sequences hybridizing to the PLF-1 probe and had to resort to looking at individual clones rather than a mRNA population. They suggested that there were probably lower amounts of PLF-1 mRNA. As shown in Table 5, mrp/plf1 encodes a legitimate protein and has correct intron/exon splice junctions. The promoter is capable of driving the expression of a cat gene (discussed in chapter 4). These results strongly suggest that this gene is functional.

The overall gene structure and preservation of the position of the splice sites and cysteine residues places MRP/PLF in the mammalian growth hormone-prolactin family. This is a large family whose members likely arose by duplication of an ancestral gene. In spite of their relatedness, members of this family are expressed in a tissue specific manner. Since the initial duplication, individual members of this family must also have duplicated. There are multiple copies of the human growth hormone gene (Seeburg, 1982) and the murine MRP/PLF gene. The MRP/PLF

gene duplication event must have occurred recently since there are only 5 amino acid changes in the coding region and on average there are 0.1 to 1.0 amino acid changes per 10^6 years. All of the known members of the murine prolactin-growth hormone family, with the exception of growth hormone which is on chromosome 11, have recently been mapped to chromosome 13 of the mouse (Jackson-Grusby et al., 1988). This clustering of the genes suggests a recent duplication event. Until the structure of more MRP/PLF genes is looked at, it is not possible to say whether the genes are exact copies of each other, in which case the DNA around the gene is probably amplified, or whether they are gene duplications. Evidence suggests that they are duplications rather than amplified since minor changes are observed in the coding region and 5' upstream region. In addition, analysis of genomic DNA by Southern blotting resulted in a number of hybridizing fragments with varying size and various degrees of hybridization. There are several other examples of gene families that arose by gene duplication including globins (Efstratiadis et al., 1980), actins and tubulins (α and β) (Firtel, 1981), histones (Hentschel and Birnstiel, 1981), immunoglobulins (Honjo, 1983) and transplantation antigens (Hood et al., 1983). In humans there is a family of "β-like" globin chains which are formed successively during embryogenesis. Until each of the individual MRP/PLF genes is characterized, it will not be known how they relate to

each other with regard to time of expression, degree of expression, and inducers required for expression.

It is interesting that each of the 5 amino acid changes in the predicted MRP/PLF proteins involves a serine or a threonine residue. Within these 5 amino acid residues, PLF-1 has 2 serines and 1 threonine, PLF-2 has 3 serines and mrp/plf1 has 2 serines. This could have profound implications if phosphorylation were involved in regulating the MRP/PLF protein, a possibility which as far as we know, no one has looked at. These altered residues, incidently, do not occur in the N-linked glycosylation sites. O-linked glycosylation, however, occurs on serine or threonine residues shortly before the protein is secreted. Although there have been no reports of O-linked glycosylation in MRP/PLF, it is possible that it may be on a small number of molecules.

Amino acid changes may also alter the conformation of the protein. A slight change may affect modifications to N-linked glycosyl groups. In fact, MRP/PLF proteins are observed on SDS polyacrylamide gels with varying degrees of glycosylation (Nilsen-Hamilton et al., 1980).

CHAPTER 4

REGULATION OF MRP/PLF EXPRESSION, PARTICULARLY EXPRESSION OF *mrp/plf1*

4.01 Abstract

Various segments of the *mrp/plf1* promoter were cloned into the pSV0*cat* expression vector. It was found that the *mrp/plf1* promoter is capable of initiating transcription in BNL cells, but it is a weaker promoter than the PLF42 promoter which is an MRP/PLF promoter identified by Linzer and Mordacq, (1987). Experimental evidence suggests there is a negative regulatory element located within a defined 65-bp segment in the promoter region of *mrp/plf1*.

Northern blotting revealed that in BNL cells, MRP/PLF expression can be positively regulated by TGF-α, β-estradiol, and possibly vitamin D3, and negatively regulated by dexamethasone. Deletions were created in the *mrp/plf1* promoter and cloned into pSV0*cat* to enable the future identification of the cis-acting DNA sequences responsible for this regulation.

4.02 Introduction

In chapter 3, the molecular anatomical details of an MRP/PLF gene were analysed. The characteristics of the primary structure and exon organization can give much insight as to how various genes are related or evolved from each other. This is exemplified in Figure 6. When one compares sequences found in the promoter region of the gene under investigation with published sequences shown to be involved in growth factor induction or tissue specificity, one has some clue as to how the gene in question might be regulated.

As discussed in chapter 3, it appeared that mrp/plf1 might be responsive to glucocorticoids. The fact that glucocorticoids, estrogen, vitamin A, vitamin D3 and TGF-α are known to be present in the placenta makes them candidates for regulating MRP/PLF expression in vivo. This hypothesis can be tested by using the Northern blotting technique to look at the effect of the various compounds on the level of MRP/PLF cytoplasmic RNA. However, since a probe for looking at an individual MRP/PLF gene is not available, the average responsiveness of the MRP/PLF gene family is observed. In addition, the Northern blotting technique only looks at changes in the levels of MRP/PLF cytoplasmic RNA and does not distinguish between the different levels of RNA regulation which may be occurring, these being transcriptional, processing, transport or stability.

The subtleties of promoter strength and expression of individual genes in a multi-gene family during processes such as development and mitogenic induction cannot be guessed and thus there is a need for an assay for the biological activity of the gene in question.

An assay which has been widely used to study the regulation of various eukaryotic promoters is based on the use of mammalian expression vectors which carry the bacterial gene chloramphenicol acetyl-transferase (*cat*). They can be propagated in prokaryotic cells and subsequently introduced into appropriate mammalian cells. The pSV2*cat* vector consists of the pBR322 origin of DNA replication, the β-lactamase gene which confers resistance to ampicillin, and segments of DNA encoding the mammalian virus SV40 early promoter, splicing and polyadenylation signals. It contains all the signals believed to be necessary for gene expression in mammalian cells. The *cat* gene is transcribed from the SV40 early promoter and the transcript is processed at the SV40 splicing and poly-adenylation signal located downstream from the *cat* gene. Following translation of the resulting transcript, the enzyme activity can be quantitated in a rapid and simple assay. The assay involves the transfer of acetyl groups from acetyl CoA by the CAT enzyme to [^{14}C]chloramphenicol. The pSV0*cat* vector is identical to the pSV2*cat* vector except that it lacks the SV40 early promoter and origin of replication. Since expression of a gene is dependent on the presence of a

functional promoter, the *cat* gene is not expressed. If a test eukaryotic promoter is inserted into pSV0*cat*, the transcriptional activity of this test promoter can be assessed by measurement of CAT enzyme activity.

In most regulated genes, cis-acting regulatory sequences are located 5' to the transcriptional start site. CAT assays provide a means of looking at the functionality of a particular promoter and its response to growth factors. This is especially important when working with a multi-copy gene family. Deletions can be made *in vitro* in the promoter region in order to fine map the cis-acting DNA sequences regulating that particular gene.

The advantage that Northern blotting has over CAT assays is that a normal situation is being examined. For the purposes of CAT assays, the promoter in question is subcloned into a vector, grown in bacterial cells and then transfected into mammalian cells. During this time, methylation patterns undoubtedly change and obviously the promoter is no longer in the same chromatin state as it is *in vivo*. These architectural changes may result in the altered binding of regulatory proteins.

4.03 The Promoter Activity of *mrp/plf1*

The *EcoR*I-*Pst*I fragment of *mrp/plf1* (Figure 9), was subcloned into the pSV0*cat* vector as illustrated in Figure 11, to give pEP*cat*. This fragment is approximately 1160 bp in length and contains about 1100 bp of 5'-flanking

sequence and 60 bp of the first exon. pEP*cat* was transfected into BNL cells using the calcium phosphate technique described in section 2.35. Cell lysates and the CAT assay were as described in sections 2.36 and 2.38. BNL cells were chosen for these experiments because they represent a cell line in which MRP/PLF is normally expressed and therefore are likely to have the transcription factors which are necessary for MRP/PLF expression.

The promoter activity of pEP*cat* was found to be very weak, only slightly higher than that of pSV0*cat* which serves as a negative control. Low level expression of a promoter could indicate that an enhancer element is missing in that construct. Upon inspection of the *mrp/plf1* 5' end sequence, GTTGGAAA was observed beginning 10 nucleotides from the *Eco*RI site. This sequence resembles the GTGGAAA consensus sequence which has been proposed for viral enhancer elements (Weiher *et al.*, 1983). It was possible that the S1 nuclease used to blunt the ends of the *Eco*RI-*Pst*I fragment had eliminated part of this sequence, resulting in the low level of promoter activity. A longer promoter fragment might therefore circumvent this problem. Unfortunately, a longer *mrp/plf1* promoter fragment is not available so the 2-Kbp *Pst*I fragment of clone 1.3 (see Figure 3), was isolated and cloned into pSV0*cat* to give a plasmid known as p2P*cat*. As explained in chapter 3, all of the genomic clones isolated in this work appear to represent the same gene so it should not matter that the

promoter segments did not originate from the same isolate. A ScaI-PstI clone known as pSPcat was generated as a negative control for this putative enhancer element. The ScaI site is located about 65 nucleotides from the EcoRI site. The sequence of the deleted region is shown in Figure 11.

Unexpectedly, the p2Pcat clone did not exhibit any increase in promoter activity over that of pEPcat while the pSPcat clone showed an approximately 10 fold increase in promoter activity. A number of transfections were done with these clones. Results within a given experiment were consistent but there was significant variability between experiments done on different days. Trends were present though, with the activities shown by pEPcat and p2Pcat always either at background level or just slightly above while pSPcat gave about 11 to 21% of the activity shown by pSV2cat (see Table 9).

4.04 Analysis of MRP/PLF Response to Growth Factors by Northern Blotting

In order to look at whether MRP/PLF expression is responsive to the various reagents discussed in section 4.02, cytoplasmic RNA was obtained from BNL cells which had been treated with these reagents. When these experiments were initiated it was not known whether BNL cells had the necessary receptors for the various factors being tested. Another mouse fibroblast line, the L cell line, has been

Figure 11 Construction of MRP-CAT Plasmids

The approximately 1160-bp EcoRI-PstI fragment and the approximately 1100-bp ScaI-PstI fragment were isolated from pES2. They were blunted with S1 nuclease and cloned into the SmaI site of pSVOcat to generate pEPcat and pSPcat. The sequence of the region which is present in pEPcat but absent in pSPcat is shown at the top of this figure.

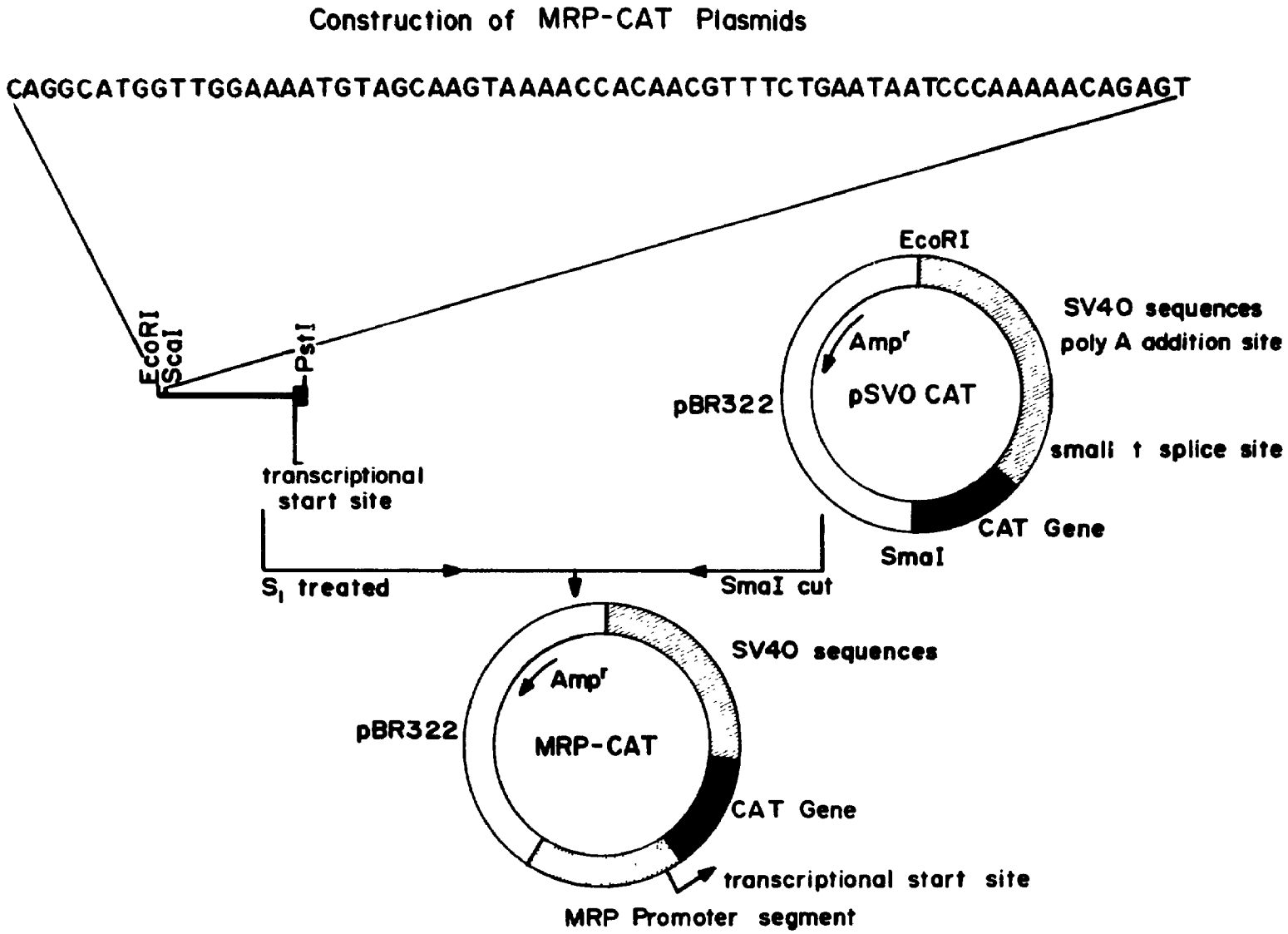

Construction of MRP-CAT Plasmids
CAGGCATGGTTGGAAAATGTAGCAAGTAAAACCACAACGTTTCTGAATAATCCCAAAAACAGAGT
EcoRI
ScaI
PstI
transcriptional
start site
EcoRI
SV40 sequences
poly A addition site
Amp^r
pBR322
pSVO CAT
small t splice site
CAT Gene
SmaI
S1 treated
SmaI cut
SV40 sequences
Amp^r
pBR322
MRP-CAT
CAT Gene
transcriptional start site
MRP Promoter segment

Table 9

Activity of the MRP/PLF Constructs

Experiment	Acetylated forms of [^{14}C]chloramphenicol as a percentage of the total [^{14}C]chloramphenicol[a]			
	pSV0cat	pEPcat	pSPcat	p2Pcat
1	0	1	---	---
2	1	2	11	---
3	2	2	21	3
4	5	---	16	2

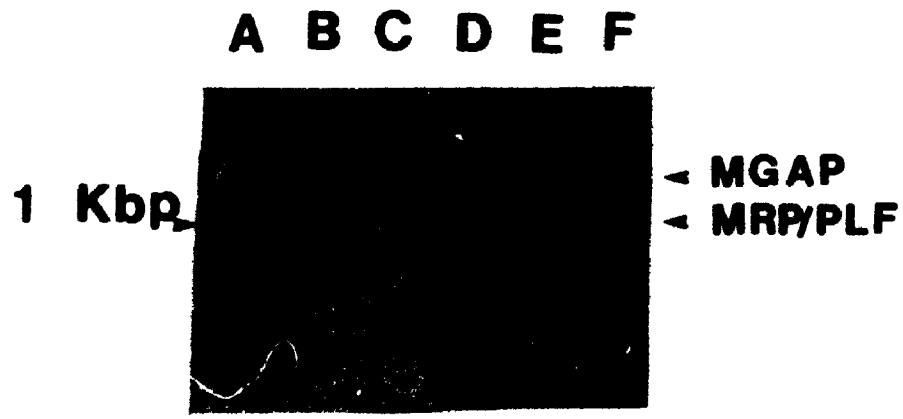
a) In all cases the percent conversion of [^{14}C]chloramphenicol to its acetylated products by lysates from cells transfected into pSV2cat was set at 100% and the above values have been adjusted accordingly.

used quite extensively for induction experiments involving glucocorticoids and estrogen, so it seemed reasonable to assume that the BNL cells might be suitable as well. As shown in Figure 12 the L cell line would not be suitable for the experiments described here because it does not normally express MRP/PLF mRNA. The MRP/PLF probings were over-exposed so that small amounts of hybridizing RNA present would be detected. There is a small amount of hybridizing RNA detected in the MEF lane but this is likely due to spill over from the BNL lane.

Large plates (150 mm) were seeded with 2 x 10^6 BNL cells in DMEM plus 3% charcoal-inactivated serum (Dobner et al., 1981). The charcoal binds small molecules such as vitamins and steroids that contain a hydrophobic portion and whose presence in the serum would interfere with induction experiments. The concentration of serum was kept at 3% so as to minimize the amount of serum present but have enough so the cells would still be growing. The estrogen induction experiments were performed in DMEM medium made up without phenol red since it has been reported that phenol red can mimic estrogen and obscure any estrogen response (Berthois et al., 1986). The DMEM medium was made to contain (200 mg/l $CaCl_2$, 400 mg/l KCl, 97.67 mg/l $MgSO_4$ (anhyd), 6400 mg/l NaCl, 125 mg/l NaH_2PO_4, 4500 mg/l D-Glucose, 584 mg/l L-Glutamine, 30 mg/l glycine, 42 mg/l L-Serine, 1X MEM amino acid solution, and 1X MEM vitamin solution). Phenol red was ommitted from this

Figure 12 Presence of MRP/PLF RNA in Various Cell Lines

Cytoplasmic RNA, 10 µg, isolated from subconfluent growing murine cell lines, was electrophoresed in 1.1% agarose formaldehyde gels and transferred to nitrocellulose. The blot was initially hybridized with oligo-labelled MRP/PLF gel-purified insert and subsequently hybridized with a control MGAP clone whose abundance is invariant in the different cell lines. Lanes: A, BNL; B, MEF; C, 3T12; D, 3T3; E, L; F, C122


A B C D E F
1 Kbp
MGAP
MRP/PLF

medium. About thirty hours after plating the cells, inducers were added directly to the plates so the final concentration was: TGF-α, 50 ng/ml; dexamethasone, 1 X 10^{-7} M; vitamin D3, 1 ng/ml; vitamin A, 1 X 10^{-5} M; β-estradiol, 1 X 10^{-7} M. These concentrations were chosen from the literature. Eighteen hours after the addition of the inducers, the cells were harvested and RNA was extracted as described in section 2.39. The RNA was analysed by Northern blotting (section 2.41). The blot was initially probed with a purified MRP/PLF cDNA probe and subsequently with a glyceraldehyde 3-phosphate dehydrogenase (MGAP) cDNA probe. MGAP is routinely used in our laboratory as a control for loading errors and RNA degradation. The results of these probings are shown in Figure 13. The autoradiograms were scanned with a laser densitometer for quantitation. The addition of vitamin A appeared to have little effect on the level of MRP/PLF cytoplasmic RNA levels. Vitamin D3 caused a small increase over control values while the addition of β-estradiol and TGF-α resulted in levels of MRP/PLF cytoplasmic RNA which are 134% and 143% respectively over those of control values. The addition of dexamethasone resulted in MRP/PLF cytoplasmic RNA levels only 56% of control values.

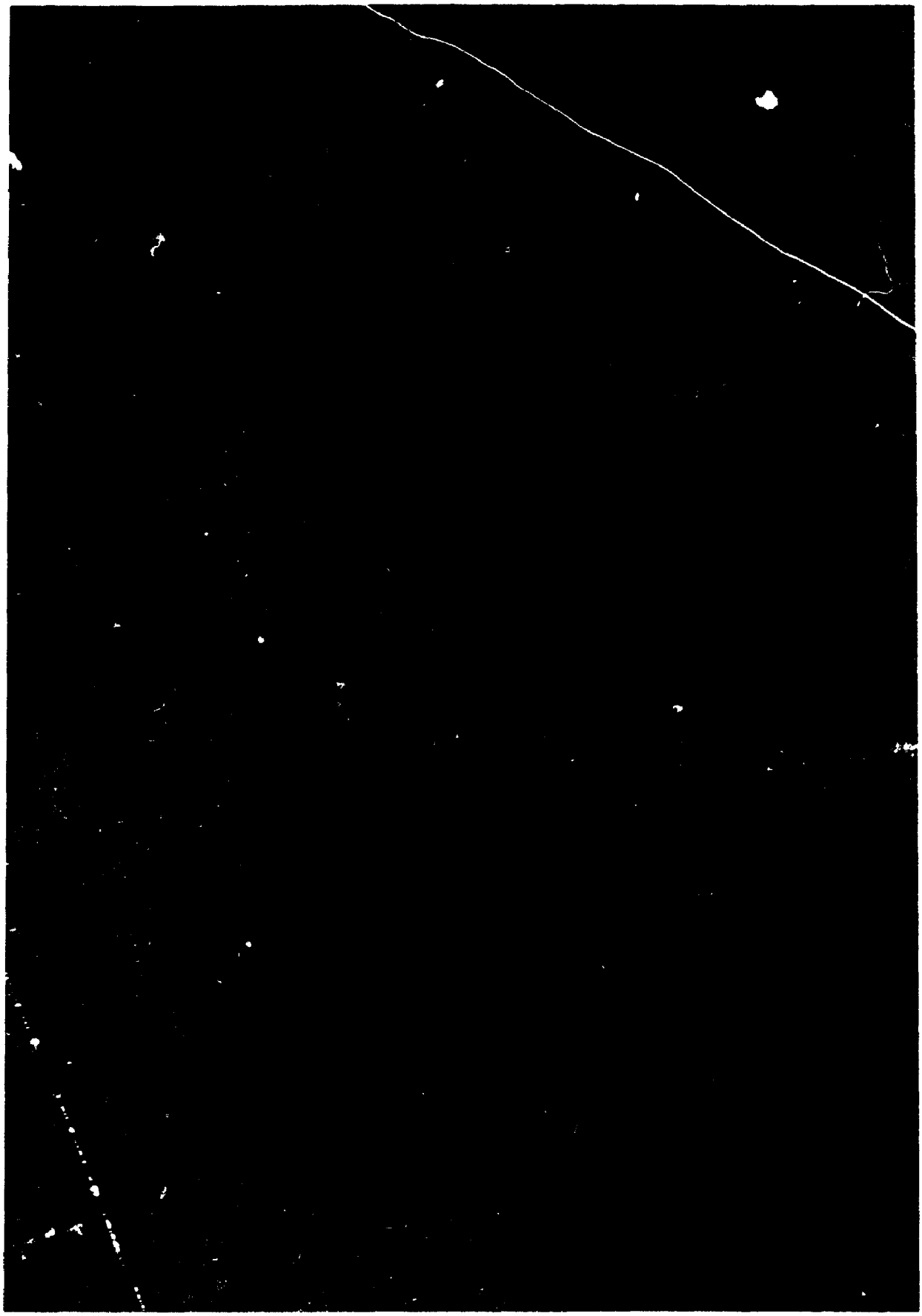
4.05 Analysis of *mrp/plf1* Response to Growth Factors by CAT Assays

The pSP*cat* clone was used in these studies since as

Figure 13 The Effect of Inducers on MRP/PLF RNA Levels in BNL Cells

Cytoplasmic RNA was isolated from subconfluent BNL cells growing in DMEM containing 3% charcoal-inactivated serum. Cells induced with β-estradiol and an uninduced control plate were grown in DMEM without phenol red (PR^-), supplemented with 3% charcoal-inactivated serum. Eighteen hours prior to harvest, inducers were added to the appropriate plates so the final concentration was: TGF-α, 50 ng/ml; dexamethasone, 1×10^{-7} M; vitamin D3, 1 ng/ml; vitamin A, 1×10^{-5} M; 17-β estradiol, 1×10^{-7} M. Ten micrograms of RNA was electrophoresed in 1.1% agarose formaldehyde gels and transferred to nitrocellulose. The blot was probed with an oligolabelled MRP/PLF gel-purified insert and then with a control MGAP clone. The results are shown in the lower panel.

The autoradiograms were scanned with a laser densitometer for quantitation. Errors in loading were determined from the MGAP hybridization and the results presented here have been appropriately corrected. The amount of MRP/PLF hybridizing RNA obtained from the control cells was set at 100%. These results are illustrated in the upper panel.



explained in section 4.03 it shows a higher level of activity than either the p2P*cat* or pEP*cat* clones. Transfections were performed as described in section 2.35. Eighteen hours before the cells were to be harvested, the inducers were added to the plates as described in section 4.04. Cells were harvested and CAT activity was assayed as described in sections 2.36 to 2.38.

Preliminary results, documented in Table 10, showed that the addition of vitamin D3 and TGF-α both had a positive effect on *mrp/plf1* promoter activity as measured by CAT assays. Vitamin A did not have much effect. The effects of dexamethasone and β-estradiol were not looked at in this experiment but work is continuing in the laboratory to look at their effect on *mrp/plf1* expression.

4.06 The P5 MRP/PLF*cat* Series of Deletion Mutants

In order to define regions in the promoter which are responsible for induction by growth factors, it is necessary to create deletions or mutations in the promoter and then check these new constructs for activity. pSV0*cat*, the vector commonly used for this purpose is a very awkward vector to work with. Fragments have to be blunted before being inserted, consequently they cannot be cut out again for purposes such as sequencing and there is no forced directionality in cloning. To circumvent these problems, a new pSV0*cat* type vector is needed with a multi-cloning site into which fragments can be subcloned.

Table 10

Preliminary Results of the Effect of Inducers on pSP*cat*

Inducer Added	Fold Induction
none	1
vitamin D3	1.6
vitamin A	0.9
TGF-α	1.5

pSP65 was chosen as the starting vector because of the orientation of the multi-cloning site with respect to the PvuII site. pSP65 contains the SP6 promoter from the SP6 phage which theoretically should not function in eukaryotic cells. To try to avoid any possible problems however, the SP6 promoter was cut out with SphI and EcoRI and the vector was subsequently re-ligated to yield a vector known as pP5. The cat gene, SV40 splice sites and poly A addition sites were obtained from pSV2cat and were inserted into pP5 to generate pP5cat. This is illustrated in Figure 14. As shown in Figure 15, pP5cat was digested with SmaI and PstI and the inserts obtained from the Bal-31 deletions (section 2.18) were cloned into this vector to give the pP5MRPcat series. A positive control for the P5 series was created by isolating the SV40 promoter from pSV2cat as a PvuII-HindIII fragment and inserting it into the SmaI-HindIII site of pP5cat. In all cases, the CAT primer (section 2.29) was used to sequence across the junction between the promoter and the cat gene. The deleted promoter regions were cut out with SacI and PstI and cloned into M13mp19 to determine the extent of the deletions. Table 11 shows the starting point of each member of this series. Unfortunately, when CAT assays were attempted with this P5 series an unacceptably high background was generated from pP5cat and it was decided that since mrp/plf1 is a weak promoter in the best of times, not to pursue this particular set of experiments.

Figure 14 Construction of pP5*cat*

1: pSP65 was cleaved with *Eco*RI and *Sph*I, then the ends were blunted with mung bean nuclease. The linearized vector, now devoid of the SP6 promoter and the *Eco*RI site in the multi-cloning site was re-ligated to yield pP5.

2: pSV2*cat* was cleaved with *Pst*I and the ends were blunted with mung bean nuclease. It was then digested with *Hind*III and the fragment containing the *cat* gene, small t splice site and poly A addition site was isolated from a low melting point agarose gel.

3: pP5 was digested with *Hind*III and *Pvu*II, dephosphorylated and ligated with the fragment described above. The resulting plasmid is pP5*cat*.

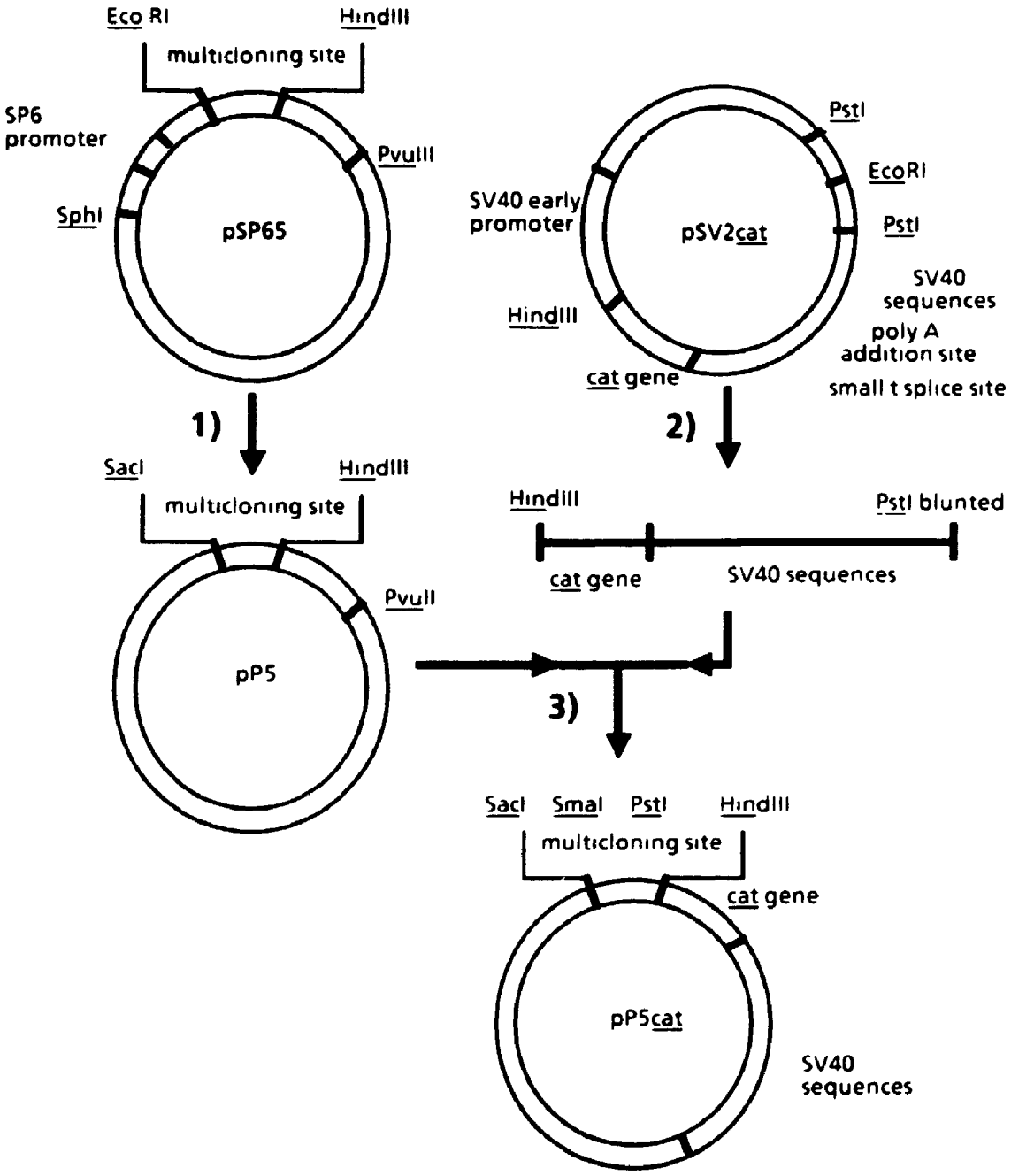

Eco RI
HindIII
multicloning site
SP6 promoter
PvuIII
SphI
pSP65
1)
SacI
HindIII
multicloning site
PvuII
pP5
PstI
EcoRI
SV40 early promoter
pSV2cat
PstI
SV40 sequences
HindIII
poly A addition site
cat gene
small t splice site
2)
HindIII
PstI blunted
cat gene
SV40 sequences
3)
SacI
SmaI
PstI
HindIII
multicloning site
cat gene
pP5cat
SV40 sequences

Figure 15 Construction of pP5MRP*cat* Series

pP5*cat* was digested with *Sma*I and *Pst*I and dephosphorylated. The Bal-31 treated DNA described in 2.18 that had a constant *Pst*I end situated just before the translational start site and varying amounts of 5' upstream sequence, was ligated to pP5*cat* to generate the pP5MRP*cat* series.

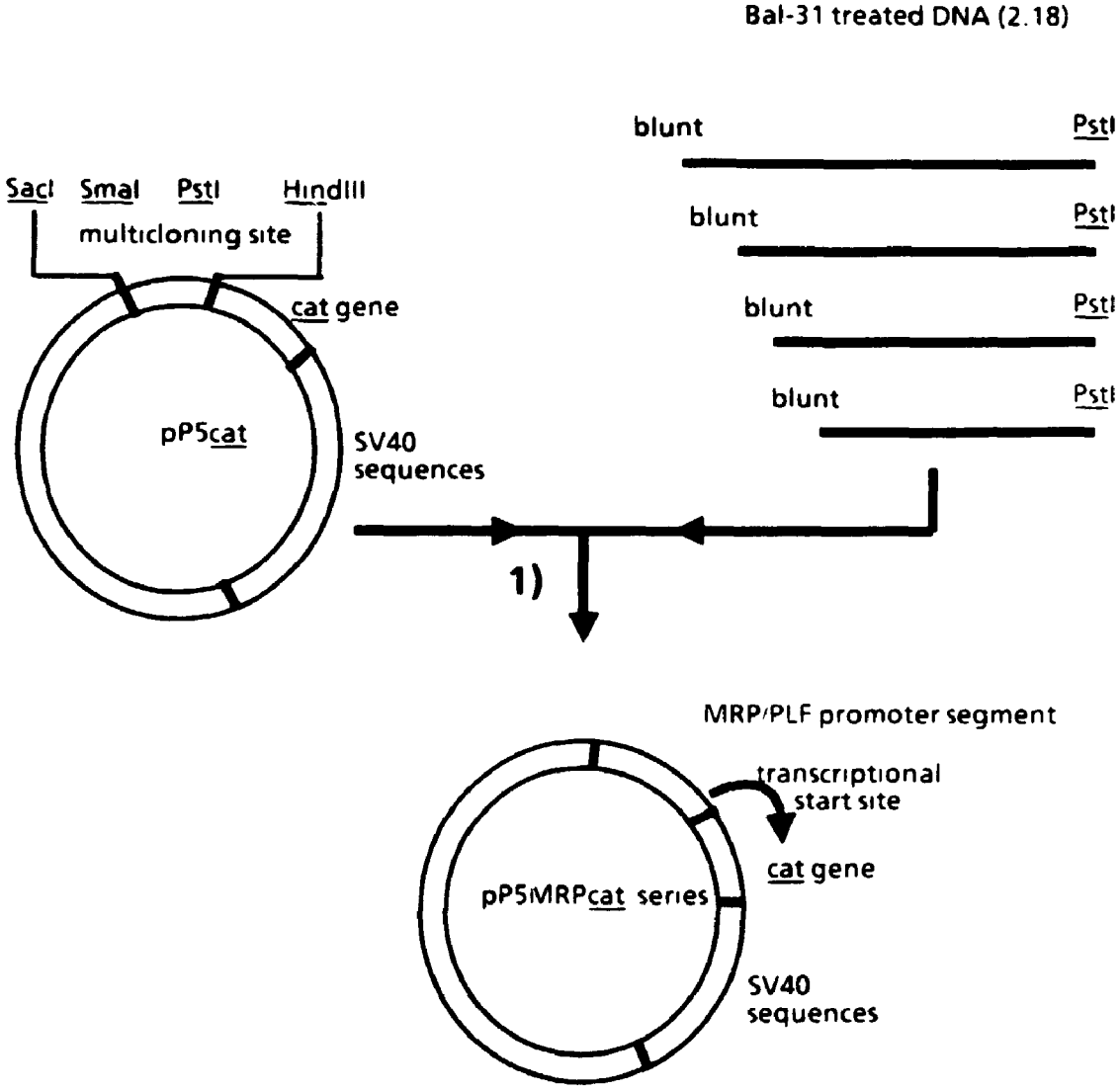

Bal-31 treated DNA (2.18)
blunt
PstI
blunt
PstI
blunt
PstI
blunt
PstI
SacI SmaI PstI HindIII
multicloning site
cat gene
pP5cat
SV40
sequences
1)
MRP/PLF promoter segment
transcriptional
start site
cat gene
pP5MRPcat series
SV40
sequences

Table 11

Characterization of the pP5MRP*cat* Series

Clone	Start Site (bp)	Relevant Features
4-13	-946	missing the putative negative regulatory element
12-2	-758	missing one GTTTTGACTT direct repeat sequence
12-6	-713	missing both GTTTTGACTT direct repeats
24-6	-93	missing an 11-bp inverted repeat sequence

4.07 *mrp/plf1* Deletion Mutants Cloned into pSV0*cat*

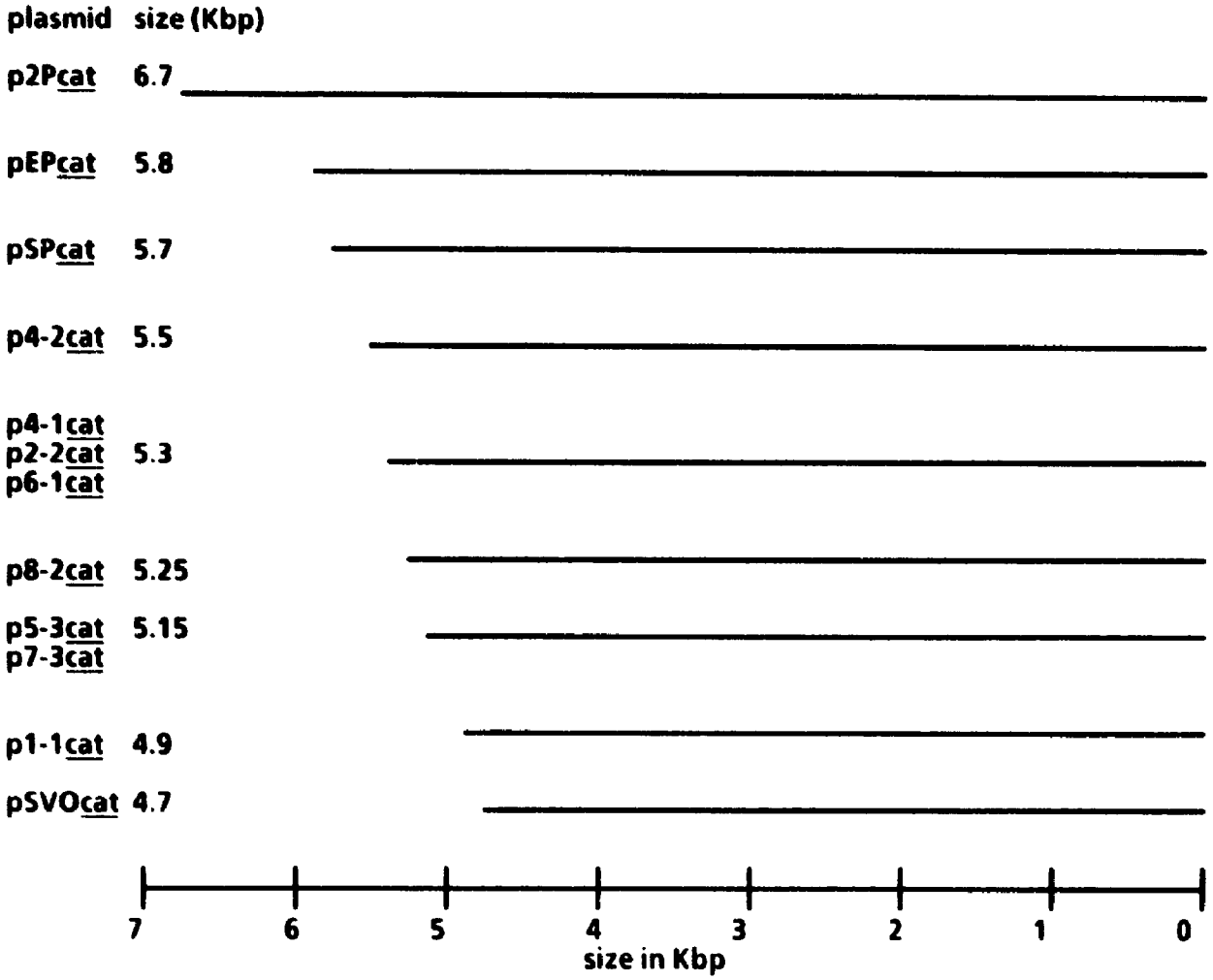
In light of the experiments described above, the fastest way of generating a set of deletions in *mrp/plf1* was to blunt the Bal-31 deletions (section 2.18) with mung bean nuclease and clone them into pSV0*cat*. The absolute end point for members of this series has not been determined but their approximate sizes, as deduced from running linearized plasmid against size markers on an agarose gel, are shown in Figure 16. They were all sequenced across the junction between the promoter and the *cat* gene and are in the correct orientation.

4.08 Discussion and Future Prospects

It is apparent from the CAT assays that the *mrp/plf1* promoter is quite weak when examined in BNL cells. In order to assess whether this was a result of the way the CAT assays were done or whether it really is a weak promoter, the PLF42*cat* vector, which contains the *cat* gene and the *Eco*RI-*Pst*I fragment of the PLF42 promoter, was kindly given to us by Dr. D. Linzer. The PLF42 promoter has been reported to be about 15 times stronger than another PLF promoter, PLF149. In addition, expression from the PLF42 promoter can be increased 20 to 40 fold in transfected cultures maintained in high serum concentrations compared to those in low serum. In contrast, the PLF149 promoter can only be induced about 4 fold under high serum conditions (Linzer and Mordacq, 1987). When the activities

Figure 16 Characterization of *mrp/plf1* Deletion Mutants Cloned in pSVO*cat*

Deletions were created in the promoter region of *mrp/plf1* by Bal-31 deletions. The deleted DNA fragments were blunted with mung bean nuclease and inserted into pSVO*cat*. Appropriate plasmids were linearized and run against size markers on an agarose gel in order to estimate the sizes shown in this figure. The constant *Pst*I site is shown on the right side while the variable 5' end is shown on the left side. The resolution of this technique was not sufficient to differentiate between similarly sized plasmids so they are shown here as groups. This is not meant to imply that they are all the same isolate. pSVO*cat*, the starting vector which does not contain an insert, is included as a reference.


plasmid
size (Kbp)
p2Pcat
6.7
pEPcat
5.8
pSPcat
5.7
p4-2cat
5.5
p4-1cat
p2-2cat
p6-1cat
5.3
p8-2cat
5.25
p5-3cat
p7-3cat
5.15
p1-1cat
4.9
pSVOcat
4.7
7
6
5
4
3
2
1
0
size in Kbp

produced from PLF42*cat* and pSP*cat* were compared under identical experimental conditions, it was found that the PLF42 promoter is about 3 to 5 times more active than *mrr*/*plf1* promoter. Under the assay conditions used in this thesis, PLF42*cat* routinely gave approximately 30 to 45% conversion compared to pSV2*cat*.

It is obvious from Table 9 that it is difficult to achieve consistent promoter activities from experiment to experiment. Within an experiment, however, duplicates were quite consistent when done. This indicates that the variability is not resulting from the experimental manipulations but rather seems to be due to the growth state of the cells or culture conditions from one experiment to another. Although it is not commented on, PLF42*cat* shows 44.5% conversion in an experiment where 7.5 µg of lysate was used and 9.1% conversion when 1.5 µg of lysate was used (Figure 2 in Linzer and Mordacq, 1987). In Figure 3 of the same paper, PLF42*cat* shows only 2.4% conversion. Since they do not mention how much lysate they used in this experiment, it is not possible to say whether this is due to experimental variability or a difference in the amount of lysate used. It seems however that when doing CAT assays, one should look for trends within a given experiment and not try to determine absolute values by averaging the results from several different experiments.

The deletion from pEP*cat* of approximately 65 bp to form pSP*cat* resulted in a 5 to 10 fold increase in the activity

of the constructs as measured by [^{14}C]chloramphenicol conversion. It is thus presumed that the removal of the 65 bp restored functionality to the mrp/plf1 promoter. This suggests that either there is something inherently wrong with the pEPcat and p2Pcat clones which prevents them from expressing the cat gene or there is a negative regulatory element within the 65 basepairs which was deleted. The junction between the promoter sequence and cat gene was sequenced in all cases and found to be as expected. This does not rule out the possibility that some small mutation or deletion occurred elsewhere in the promoter fragment or in the cat gene during the cloning process.

The presence of a negative regulatory element is an interesting possibility. This would not seem unreasonable when one considers that there are a number of instances where MRP/PLF is not expressed. During embryonic development, MRP/PLF is under the control of regulatory processes that involve the repression (before day 9 of gestation) and activation (day 9 and 10 of gestation) of transcription. This gene family is expressed specifically in the placenta and not in other tissues. It is also inducible in a number of cell lines in culture by the addition of mitogens such as serum, EGF and FGF. It should be remembered that gene activity depends on the sum of all regulatory elements whether they are positive or negative. An observation which initially does not seem to fit in with these results is that PLF42cat which also contains the EcoRI-PstI

promoter fragment shows much higher activity relative to the pEP*cat* clone. Since the sequence of the region from -1100-bp to -578-bp of the PLF42 promoter, which includes the putative negative regulatory element, has not been reported, it is not possible to say how similar these two promoter regions are. Alternatively, this inconsistency can be explained by the finding that negative regulatory elements can strongly repress weak transcriptional units but only weakly repress strong transcriptional units (Baniahmad *et al.*, 1987). The PLF42 promoter is a stronger promoter than *mrp/plf1* and perhaps it is able to compensate for the negative effect of a potential negative regulatory element.

Although experiments with cycloheximide have shown that MRP/PLF is not superinducible and requires prior protein synthesis for its expression, its level can be increased by cycloheximide once it is induced (Denhardt *et al.*, 1986). This suggests that MRP/PLF is also regulated by proteins which stabilize mRNA or by labile factors which perhaps modify pre-existing cellular factors. It is possible to imagine that a negative regulatory factor becomes transiently modified during the induction process to allow MRP/PLF gene expression.

The aspect of negative regulation has not been looked at in as much detail as positive regulation by enhancer elements. Negative regulatory elements have been reported in several genes including the human β-interferon gene

(Goodbourn et al., 1986), the chicken lysozyme gene (Baniahmad et al., 1987), the rat growth hormone gene (Larsen et al., 1986) and the rat insulin gene (Laimins et al., 1986). There is a sequence in the EcoRI-ScaI region at position -1081 to -1071 with a sequence of AGCAAGTAAAA that is very similar to the multiple repeats of the rat insulin silencer. To confirm that there really is a negative response element in the 65 bp separating the EcoRI and ScaI sites, this region will have to be placed upstream of another promoter to see if it can confer negative regulation.

There are a number of reasons why the inductions seen in Figure 13 are not as great as might be expected. BNL cells may not be very responsive to the factors used or the cells may not have been depleted of their intracellular pools of these factors. The amount of compound to add was determined from the literature rather than from a dose response curve. A time course which would have allowed the determination of the optimal time for exposure to the various compounds was not established. Instead, cells were exposed to the factors for 18 hours, a period of time which seems to be about optimal for MRP/PLF induction by other growth factors. These results more than likely are a true indication that MRP/PLF is regulated by these factors since firstly, all results were normalized to a control mRNA whose relative abundance should not be affected by the growth state of the cells and secondly, for those compounds

tested, the CAT assays showed a similar response. Further evidence that these compounds are effecting the transcription of MRP/PLF genes was gained when their effect on the PLF42cat plasmid was examined. TGFα and estrogen had a positive effect while dexamethasone had a negative effect on the amount of assayable CAT enzyme (data not shown). As will be discussed later, these results are reasonable when considered in the context of what is known about other genes. It is important to remember that changes in the level of a given cytoplasmic RNA and the effects of steroid hormones on gene expression are not restricted to transcriptional events. Estrogen not only increases transcription of the vitellogenin gene in Xenopus laevis liver but it also affects the stability of the corresponding mRNA (Brock and Shapiro, 1983). Glucocorticoids are capable of increasing transcription of the human growth hormone gene and enhancing the stability of the hGH mRNA (Paek and Axel, 1987). CAT assays can be useful in determining whether increases in cytoplasmic RNA abundance result from increased transcription of the gene. Preliminary evidence suggests that vitamin D3 and TGF-α do increase transcription of mrp/plf1. The effect of dexamethasone and β-estradiol have yet to be determined. These results are interesting as it appears as if the mrp/plf1 promoter, which is a weak promoter, reflects the results seen by Northern blotting which are an average of the responses of all the MRP/PLF genes. This suggests that all of the MRP/PLF genes are likely

regulated in the same manner. Some may be more or less responsive to certain reagents, for example PLF42 is more responsive to serum induction than PLF149, but they probably all respond to some degree when presented with a given stimulus.

The RNA corresponding to a cDNA clone isolated from mouse placenta was identified in BALB/c 3T3 cells and the RNA corresponding to a MRP/PLF cDNA clone isolated from 3T3 cells was identified in the mouse placenta. This evokes the idea that at least in the case of 3T3 cells, the use of tissue culture for MRP/PLF expression studies is relevant to *in vivo* expression (Wilder and Linzer, 1986). In this study, it has been assumed that the BNL line also reflects *in vivo* expression but it would be interesting to try using trophoblast cells for some of the CAT assays. The *mrp/plf1* promoter would then be in its natural environment and a true determination of the promoter strength, importance of the putative negative regulatory element and the factors affecting the *in vivo* regulation of MRP/PLF expression could be determined. Nevertheless, bearing in mind the limitations of the tissue culture system, it is tempting to try to extend the results obtained from BNL cells to a model to explain how MRP/PLF might be regulated *in vivo* in the placenta.

It had been shown previously that the expression of MRP/PLF is induced by EGF (Nilsen-Hamilton *et al.*, 1980). In the present study, TGF-α was looked at because it shares

structural homology and binds to the same cell surface receptor as EGF. EGF receptors have been found on several embryonic tissues and in placenta including mouse trophoblast cells (Adamson and Meek, 1984). It has been reported that TGF-α is transiently expressed during fetal development with the highest levels in day 7 to 9 fetuses (Wilcox and Derynck, 1988). Recently, *in situ* hybridization has been used to show that TGF-α is located in the fetal part of the placenta as well as in a number of other organs Since MRP/PLF is also located in the fetal part of the placenta and is maximally induced on day 9 and 10 of gestation, right after the expression of TGF-α, it is likely that TGF-α is at least in part responsible for MRP/PLF expression. This theory is supported by the results presented here that in the BNL cell system, MRP/PLF expression is induced by the addition of TGF-α to the culture medium. Another source of material which can bind to EGF receptors is the EGF from the maternal circulation. Most of this EGF is transported to the placenta and uterus rather than to the fetus, Popliker *et al.*, 1987, so it too might be enhancing MRP/PLF expression.

The effect of estrogen on MRP/PLF expression was looked at because it is produced by the placenta in proportion to the duration of the pregnancy. In this study, estrogen was shown to increase the level of MRP/PLF RNA expression. The expression of other members of the GH-PRL family has previously been reported to be increased in response to

estrogen. Estrogen is also known to increase the expression of TGF-α, PDGF, and IGF-I (Murphy et al., 1987). A cascade mechanism is thus beginning to emerge in which estrogen increases the amount of TGF-α which then induces MRP/PLF expression. Unless estrogen is also increasing the amount of TGF-α in BNL cells, it appears as if it can act directly on MRP/PLF genes or with MRP/PLF mRNA. It would be interesting to add both TGF-α and estrogen to cells and see if their effects are additive.

The effect of the synthetic glucocorticoid dexamethasone was looked at because as mentioned in chapter 3, 2 glucocorticoid binding sites were identified in the promoter region of mrp/plf1. Glucocorticoid receptors are widespread so it is reasonable to assume that they might also exert their effects in the placenta. It was found that MRP/PLF expression decreased in response to the administration of glucocorticoids to BNL cells. This is reminiscent of another member of this family, prolactin, whose level of expression also decreases in response to glucocorticoids (Tashjian et al., 1970). Growth hormone on the other hand is induced by glucocorticoids (Evans et al., 1982) while IGF-I, which mediates many of the biological effects of GH, is reduced (Adamo et al., 1988).

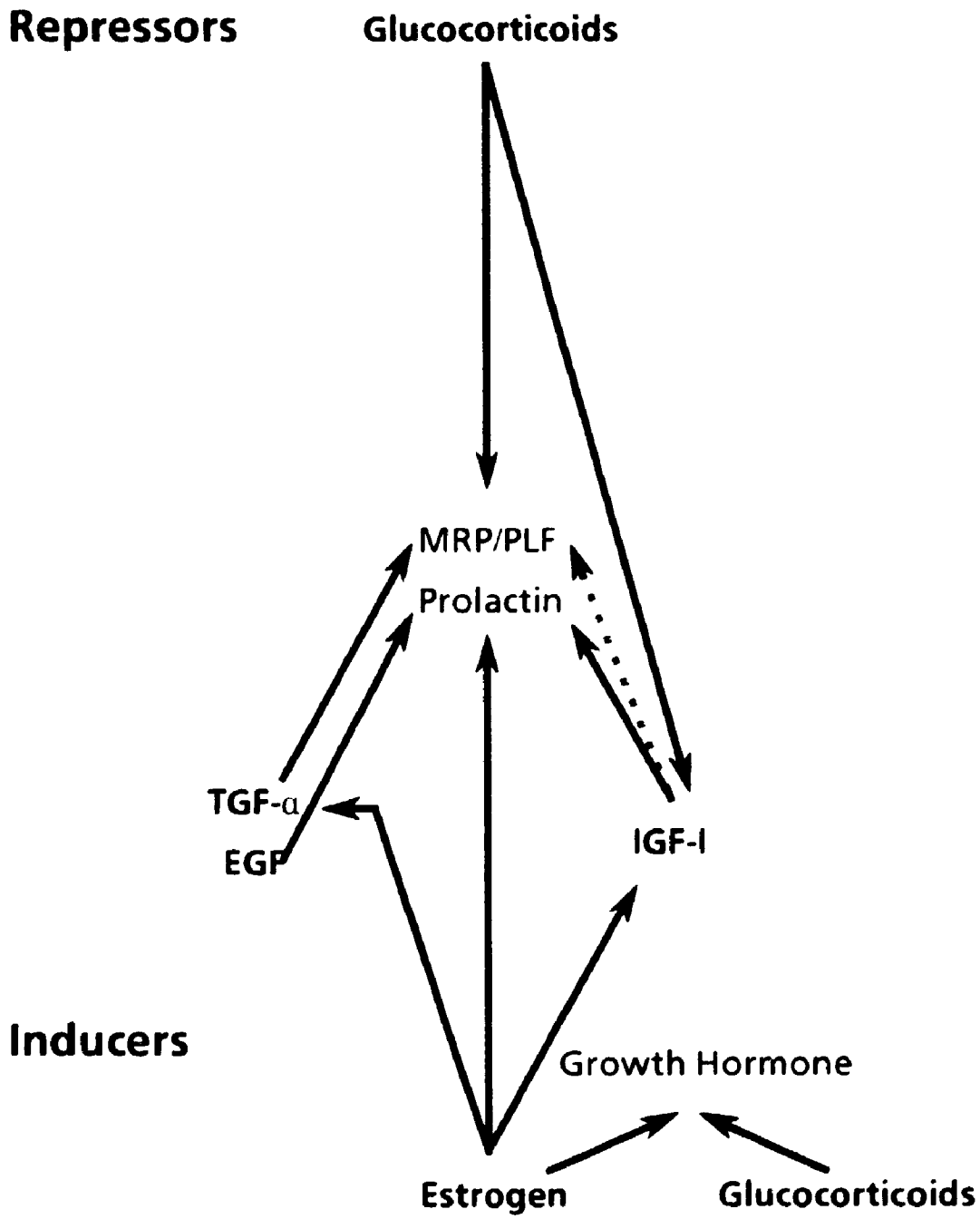
DNase I and exonuclease III footprinting experiments in combination with gene transfer experiments of deletion mutants into appropriate cells revealed that the binding site for the glucocorticoid receptor in the rat pro-opio-

melanocortin gene, which is negatively regulated by glucocorticoids, is the same as the binding site identified in glucocorticoid-inducible genes (Drouin et al., 1987). CAT assays and deletion mutants of the rat prolactin gene were used to identify a region containing a glucocorticoid down-regulated element. Again, a sequence similar to the glucocorticoid regulatory element was identified (Somasekhar and Gorski, 1988). It is possible that the positive and negative effects of glucocorticoids are mediated through the same response element indicating that the regulation of a response element can be very complex involving a precise interplay of various transcription factors.

A model depicting how all these reagents may interact is shown in Figure 17. When it is considered that MRP/PLF is expressed at a specific time in a specific tissue, it should not be surprising that its regulation is complex. No doubt it is more complex than depicted but it is possible that some of the regulators of MRP/PLF gene expression may share a common step, for example the induction or modification of a specific regulatory protein. Prolactin is also regulated tightly. The relative level of PRL RNA increases in response to estrogen, thyrotropin-releasing hormone, calcium, EGF and vasoactive intestinal peptide. The relative level of PRL RNA decreases in response to the dopamine agonist ergocryptine and dexamethasone (Shull and Gorski, 1986). A fine tuning mechanism can be achieved when a gene responds to both

Figure 17 A Proposed Model for the Regulation of Expression of Members of the GH-PRL Family

TGF-α and estrogen are shown in the lower part of the figure to indicate that they are inducers of expression. Glucocorticoids are shown in the top part of this figure to indicate that they are repressors of expression. The effect of these compounds (shown in bold type) on other members of the GH-PRL family is indicated. Another growth factor, IGF-I is predicted to increase MRP/PLF expression but since it has not been proven, this is indicated by a dotted line. No attempt has been made to indicate the level at which these compounds are affecting expression.


Repressors
Glucocorticoids
MRP/PLF
Prolactin
TGF-α
EGF
IGF-I
Inducers
Growth Hormone
Estrogen
Glucocorticoids

negative and positive regulators.

A curious point derived from the literature in this field is that IGF-I and PRL seem to be regulated in much the same manner as MRP/PLF. This is not surprising in the case of PRL since it is in the same family as MRP/PLF. The interesting aspect of IGF-I though, is that it has been shown *in vitro* to stimulate production of placental lactogen from human placental explants (Bhaumick *et al.*, 1987) and synthesis and release of PRL from human decidual cells (Thrailkill *et al.*, 1988). Since IGF-I in combination with PDGF and EGF has been shown to increase MRP/PLF expression *in vitro* (Denhardt *et al.*, 1986), and since as explained above, other members of the PRL-GH family are induced by IGF-I, it would be interesting to see if the addition of IGF-I to trophoblasts (the cells where MRP/PLF is expressed *in vivo*) has any effect on MRP/PLF synthesis or release. CAT assays could be used to identify an IGF-I responsive element in the PRL, MRP/PLF and PL genes. It has been shown that glucocorticoids reduce the steady state levels of IGF-I mRNA in cultured rat glial and neuronal cells (Adamo *et al.*, 1988). Perhaps glucocorticoids have a dual function in the regulation of MRP/PLF expression; they act directly on the gene and also act on IGF-I.

The long term goal of this project is to be able to define regions in *mrp/plf1* responsible for the regulation of its expression under given circumstances. Since MRP/PLF is apparently responsive to several growth factors and ste-

roids including serum, PDGF, EGF, FGF, TPA, TGF-α, TGF-β, estrogen and dexamethasone, it is a good model gene to use for the study of any of these compounds. It is also useful for looking at tissue specific expression and developmental regulation. As regulation usually occurs in the 5' region of a gene, deletion mutants were made in this region (section 4.07), which differ in size from each other by approximately 200 bp increments so that CAT assays can be used to define a region within approximately 200 bp which is responsible for induction by TGF-α for example. Once a region of 200 bp has been defined, it could be narrowed down by the use of further deletion mutants or by linker-scanner mutations (Haltiner *et al.*, 1985).

As discussed in section 4.06, an attempt was made to construct a vector which would be easier to work with. This vector, however, resulted in an unacceptably high background for CAT assays. The background is probably due to the generation of a cryptic promoter somewhere within the vector construct during the cloning procedure so that transcription is initiated within the plasmid and proceeds through the *cat* gene resulting in assayable CAT activity. Another problem with this vector is that the plasmid origin of replication relative to the *cat* gene is opposite that in pSV0*cat*. While it is not readily apparent what problems this could cause, it is perhaps somehow interfering with the normal regulation of *cat* gene expression. This problem could possibly be alleviated by cloning a transcriptional

termination signal, such as the UMS transcription terminator, before the *cat* gene (or before the promoter one is interested in studying) so that any spurious transcripts arising within the vector will not affect expression of the *cat* gene (Wood, 1984., McGeady, 1986). It would also be possible to release the inserts characterized in Table 11 by cleaving with *Sac*I and *Pst*I. After blunting, they could be cloned into pSV0*cat*.

CHAPTER 5

REGULATION OF EXPRESSION OF MRP/PLF IN MORTAL AND IMMORTAL MOUSE CELLS

5.01 Abstract

The immortalization of cell lines is regarded as one step in the transformation process. Little is known about the mechanisms regulating spontaneous immortalization, under certain culture conditions, of rodent cell lines. MRP/PLF is a rare example of a gene which is expressed in the immortal 3T3 line but not in the MEFs from which it is derived. Although there is no evidence that MRP/PLF is directly involved in the immortalization process, it is conceivable that whatever regulatory processes are involved in its expression are also responsible for the expression of the immortal phenotype. Results presented here show that the increased expression of MRP/PLF in 3T3 cells is not accompanied by any major changes in the genome such as amplification of the gene, generation of new DNase I hypersensitive sites or methylation. Instead, it appears to result from the change in expression of a regulatory protein, an observation consistent with the theory that immortalization is due to a mutation. This regulatory protein could be involved with the post-transcriptional stabilization of MRP/PLF transcripts.

5.02 Introduction

Normal mammalian cells have a finite reproductive life span in culture; in other words, they are mortal. When the cells are placed in culture, the population initially grows rapidly but then exhibits a smooth decline in growth rate indicative of a steadily decreasing growth fraction (Todaro and Green, 1963). This loss of growth potential under serial passage conditions is referred to as senescence or crisis and it may represent programmes such as terminal differentiation or ageing. In contrast, when cells from malignant tumours are grown *in vitro*, they often display an infinite reproductive life span and are said to be immortal. The acquisition of the immortal phenotype seems to be a necessary step in the process of transformation and as a result, it is relevant to the cancer problem.

Several oncogenes have been shown to be able to confer the immortal phenotype on rodent fibroblasts. Primary rat fibroblasts could be rendered tumourigenic by combinations of a *myc* gene and a *ras* gene (Land *et al.*, 1983. Ruley, 1983). A mutant form of the cellular p53 oncogene was able to immortalize rat embryo fibroblasts which could then be transformed with activated Ha-*ras* (Rovinski and Benchimol, 1988). Primary rat embryo fibroblasts transfected with the polyoma virus large T antigen were shown to become immortal and could then be made tumourigenic with the gene encoding viral middle T protein (Cuzin, 1984). Similarly, the *E1a* gene of adenovirus is able to immortalize cells of primary

cultures but the E1b gene is required in addition to completely transform the cells (Van den Elsen et al, 1982).

On occasion, rodent fibroblasts are able to spontaneously generate immortal but untransformed cell lines. It has been shown with mouse cells that the culture conditions employed during the establishment procedure influence the properties of the resulting cell lines (Todaro and Green, 1963). When the cultures are transferred according to a specified regime, they show a progressively declining growth rate which after about 15 to 30 generations begins to rise again and reaches a level similar to that when the culture was initiated. Soon after the increase in growth rate, the culture which started out as predominantly diploid becomes heteroploid. This change in ploidy apparently occurs within 2 or 3 generations. Todaro and Green were able to conclude from their results that the establishment of cell lines was not due to a population change whereby cells able to survive in culture outgrew the rest of the population but rather seemed to result from new growth properties acquired by the cells as they were grown in culture. A direct fluctuation analysis was used to show that the frequency with which cultured Chinese hamster cells became spontaneously immortalized was 1.9×10^{-6} per cell per generation. Permanent cell lines were established only when the primary culture contained more than 10^6 cells. These data suggest that immortalization is a rare

heritable event which occurs *in vitro* and is associated with a mutation (Kraemer *et al.*, 1986). This mutation might be induced by some environmental factor such as the type of serum used, the concentration of oxygen in the cultures and exposure to low intensity fluorescent room lights during routine handling of the cells (Sanford and Evans, 1982). All of these agents are able to produce DNA damage and it is possible that the postulated mutation event, resulting in immortality, arises because of an impaired capacity to repair this damage.

It would be of interest to define the mechanism which allows the establishment of immortality. As mentioned, the seemingly unrelated *myc*, p53, large T antigen and *E1a* genes are all able to confer immortality to rodent fibroblast cells in culture. This suggests that the expression of these genes results in the induction of the same primary molecular event. A possible way of identifying this event would be to try to understand the mechanisms involved in the regulation of genes which are expressed only in immortal cells. If the initial mutation or activation event involves a regulatory protein analogous to those described in chapter 3, then it is likely that several genes involved with the expression of the immortal phenotype will be regulated in the same manner. A gene that is suitable for this type of experiment is MRP/PLF. Neither the MRP/PLF protein nor the MRP/PLF mRNA are found in mouse embryo fibroblast cells (Parfett *et al.*, 1985). If the

primary culture of these cells is plated at 3 x 10^6 cells per 50 mm plate, allowed to reach confluence and then transferred every 3 days at 12 x 10^5 cells per plate, a 3T12 cell line is generated which is not contact inhibited and does not express MRP/PLF. If the primary culture is initially treated as described but passaged every 3 days at 3 x 10^5 cells per plate, a permanent 3T3 line emerges. The 3T3 line, as originally described by Todaro and Green, is immortal, heteroploid, contact inhibited, can grow at low cell densities and does not form tumours in mice. It also expresses MRP/PLF mRNA and protein. Cells which are passaged in this manner begin to secrete MRP/PLF as they are coming out of crisis (Edwards et al., 1987). The ability of the immortal 3T3 cell line to express MRP/PLF could result from an increase in gene copy number, a change in the methylation of the genes, the acquisition of DNase I hypersensitive sites which are correlated with active genes, or a change in a regulatory protein. The work in this chapter addresses these possibilities.

5.03 Chromatin Structure of MRP/PLF in MEF and BNL Cells

DNase I is an endonuclease which can be useful in determining the chromatin structure around a gene because it shows little DNA sequence specificity. Active chromatin is preferentially digested by DNase I, and in general the region of sensitivity occurs over a region of several

kilobases beyond the transcriptional start site. DNase I hypersensitive sites are regions of the chromatin that are particularly sensitive to the endonuclease and can be identified by digesting nuclei with DNAse I so an average of one cut is introduced in a region bordered by a restriction site of interest.

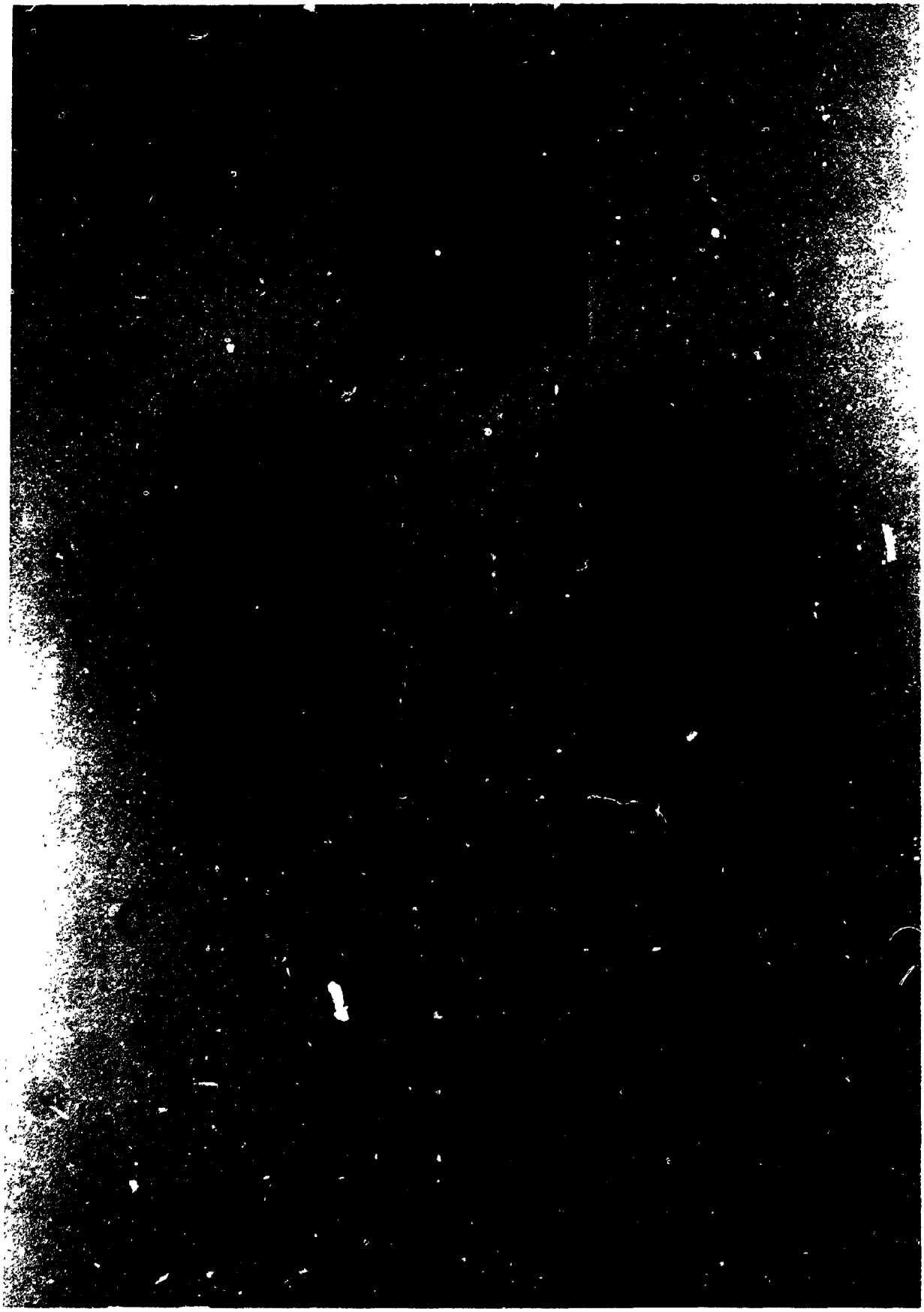
As previously stated, the BNL cell line is an immortal mouse cell line which expresses MRP/PLF while the MEFs are a mortal cell line which do not express MRP/PLF. Since nuclei of BNL cells were available, these were used to initially work out conditions. Figure 18A is an ethidium bromide stained agarose gel of DNA isolated from nuclei treated with increasing amounts of DNase I to illustrate the effects of DNase I on the size distribution of DNA. These DNA samples were treated with the restriction endonucleases PvuII and PstI and subjected to the technique of Southern (2.16). The filter was hybridized with the EcoRI-PstI probe and the result is shown in Figure 18B. Both halves of 18B were from the same autoradiogram. It is believed that the different hybridization signal intensities are a result of uneven transfer of the DNA to the nitrocellulose since a photograph before transfer (not shown) showed that equal amounts of digested DNA was loaded in all lanes. The sizes of the bands were determined by comparison with lambda HindIII size markers which were run in parallel. The normal PstI band hybridizing to the probe has a size of 2 Kbp while there are 2 PvuII bands hybrid-

Figure 18 **Mapping DNase I Hypersensitive Sites in BNL Cells**

A: Ethidium bromide-stained 1% agarose gel of DNA isolated from BNL cell nuclei which had been treated with different amounts of DNase I. Lanes: A, 5 µg/ml DNase I; B, 1 µg/ml DNase I; C, 0.1 µg/ml DNase I; D, no DNase I

B: 6 µg aliquots of the same DNA samples were digested to completion with PstI or PvuII, fractionated on a 1% agarose gel and transferred to nitrocellulose by the method of Southern. The filter was hybridized with an EcoRI-PstI probe, which encompasses part of the mrp/plf1 first exon and an additional 1100 bp upstream (see part C). Results of the PvuII digestion after probing are shown on the left side while the gel on the right side represents results of the PstI digestion. The location of the relevant HindIII markers is indicated in the middle of this figure. Bands appearing due to the presence of DNase I hypersensitive sites are indicated by arrows.

C: Proposed gene structures and position of the hypersensitive sites of the 2 different MRP/PLF genes which were identified in these studies. Gene 1 appears to correspond to clone 1.3 (see Figure 3).



izing to this fragment; one is approximately 4.3 Kbp and the other is approximately 6.7 Kbp. These two bands presumably represent different MRP/PLF genes (see 5.06). There is a single DNase I hypersensitive site picked up with this probe following the PstI digestion and 2 discernable sites following the PvuII digestion. As explained in the introduction to this chapter, nuclei are digested with differing amounts of DNase I such that one of the concentrations used will be optimal for introducing an average of one cut within the region of interest. In order to map the DNase hypersensitive site, restriction enzymes whose sites are known are chosen, which encompass the DNase I hypersensitive site. The presence of smaller hybridizing bands in the DNA isolated from nuclei digested with DNase I indicates the presence of DNase I hypersensitive sites. The use of more than one restriction enzyme and comparison of the size of the DNase I hypersensitive sites with known size markers enables the DNase I sites to be mapped.

As with many other experiments described in this thesis, there is difficulty in determining which gene is responsible for the results observed. Obviously the large fragment is giving the large sub band. If it is assumed that the 4.3-Kbp fragment is responsible for the 3.4-Kbp sub band and that the same hypersensitive site is being observed in both the PstI and PvuII digestions, then this site must be mapping approximately 100 to 200 bases away from the PstI site as illustrated in Figure 18C. When MEF

cells were examined for the presence of this site, they also appear to have it although it is difficult to determine whether the relative intensities are the same (see Figure 19). The BNL cells and MEF cells were digested in parallel in this experiment and it appears as if the digestion of the chromatin with DNase I did not work as well in this experiment as in that shown in Figure 18 hence the DNase hypersensitive sites are difficult to observe. A longer exposure of the autoradiogram was attempted but the background also increased. Under these conditions however, the DNase I hypersensitive site appears to be equivalent in both the MEF and BNL cells.

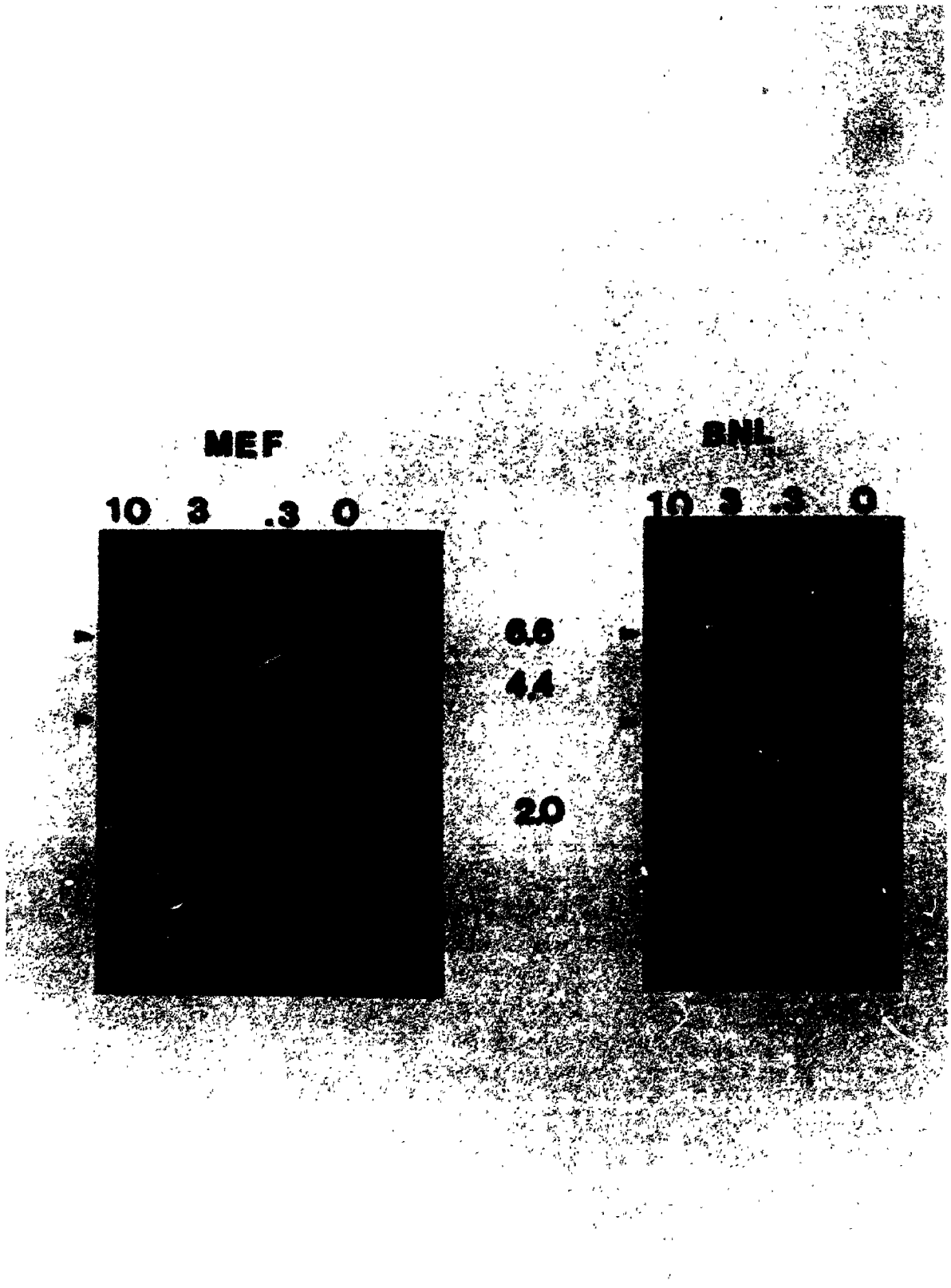
5.04 Methylation Status of MRP/PLF Genes

In eukaryotic DNA, approximately 3 to 5% of the cytosine residues have been methylated to 5-methylcytosine. Usually the methylated cytosine is found in a mCG doublet. The function of the methylated cytosine residue is not known but there is evidence that specific cytosines are more commonly methylated in the 5' flanking regions of inactive genes. It was of interest to look at the methylation status of the MRP/PLF genes to see if it differed in the mortal MEFs and immortal 3T3s.

MspI and HpaII are isoschizomers which cleave CCGG sequences. HpaII will only cleave CCGG and not CmCGG while MspI will cleave CCGG and CmCGG. The use of this pair of enzymes allows one to determine whether a given CCGG site

Figure 19 **Comparison of DNase I Hypersensitive Sites in MEF and BNL Cells**

5 µg aliquots of DNA isolated from MEF cell nuclei and BNL cell nuclei treated with differing amounts of DNase I were digested to completion with PvuII and treated as described in Figure 18. The left panel of this figure shows DNA isolated from MEF cells which had been treated with 10, 3, 0.3, or 0 µg/ml DNase I as indicated. The right panel of this figure shows DNA isolated from BNL cells treated with 10, 3, 0.3 or 0 µg/ml DNase I. DNase I hypersensitive sites are indicated with arrows.


MEF
10 3 .3 0
BNL
10 3 .3 0
6.6
4.4
2.0

is modified by methylation.

Genomic DNA was isolated and digested with MspI and HpaII as described in 2.45. The blot was probed with a nick-translated full-length MRP/PLF cDNA probe. As shown in Figure 20A, results looked promising. It appeared that MRP/PLF genes were highly methylated in both the 3T3 and MEF cells as indicated by the methylation sensitive HpaII digestions and a difference was observed in the banding patterns, indicating a change in the methylation pattern of MEFs and 3T3s. In subsequent experiments, in which the DNA was the same but the batch of HpaII was different, this difference was not observed. Figure 20B shows a typical result following HpaII digestion of DNA from the expressing BNL and 3T3 cell lines and non-expressing MEF and C122 cells. Essentially no differences in the banding patterns are seen when 20A and 20B are lined up with molecular weight markers; all of the cell lines seem to have the band originally only seen in the 3T3 cells. It is likely that in the original experiment the HpaII digestion of MEF DNA did not go to completion even though the lambda DNA which was digested in parallel had gone to completion (see 2.45). The MspI sites mapping in mrp/plf1 and the identification of some of the bands are shown in 20C.

5.05 RNA Mapping MRP/PLF Transcripts

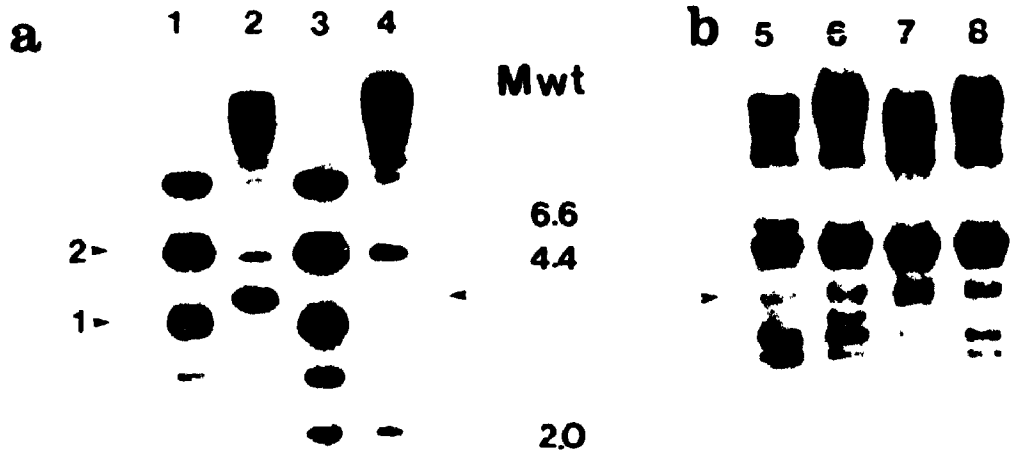
Nuclear run-on experiments suggested that MRP/PLF genes were transcribed to an equal extent in MEF and 3T3 cells

Figure 20 Methylation Status of MRP/PLF Genes in Mouse Cell Lines

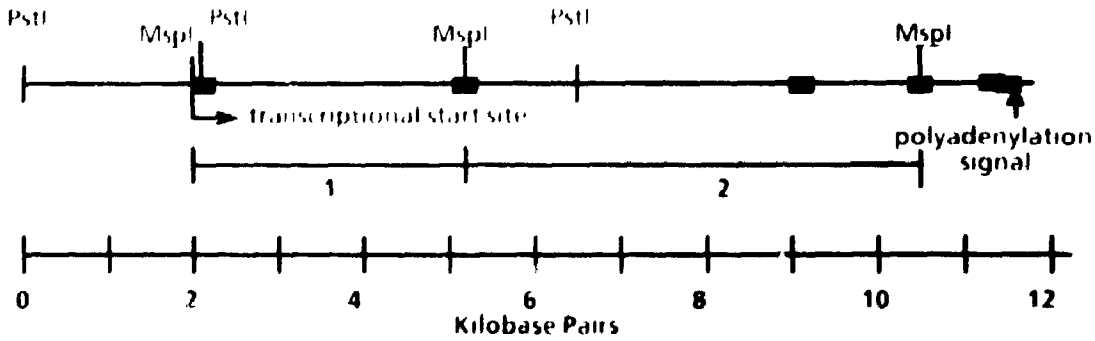
a: DNA from actively growing MEF and 3T3 cells was digested with HpaII and MspI. 6 µg of digested DNA was electrophoresed in a 1% agarose gel and transferred to nitrocellulose. The blot was probed with purified nick-translated MRP/PLF cDNA insert. Lanes: 1, MspI digested 3T3; 2, HpaII digested 3T3; 3, MspI digested MEF; 4, HpaII digested MEF

b: A typical result of subsequent experiments involving HpaII digestion. In the case of MEF and 3T3, the DNA was the same as used in panel a. Blots a and b are lined up according to molecular weight markers and the band in question is indicated with an arrow. Lanes: 5, C122; 6, BNL; 7, 3T3; 8, MEF

c: The approximate location of MspI sites in mrp/plf1 are shown. The fragments numbered 1 and 2 correspond to the bands numbered 1 and 2 in panel a.


a
1
2
3
4
Mwt
6.6
4.4
2.0
2►
1►
b
5
6
7
8

c


PstI
MspI
PstI
MspI
PstI
MspI
transcriptional start site
polyadenylation signal
1
2
0
2
4
6
8
10
12
Kilobase Pairs

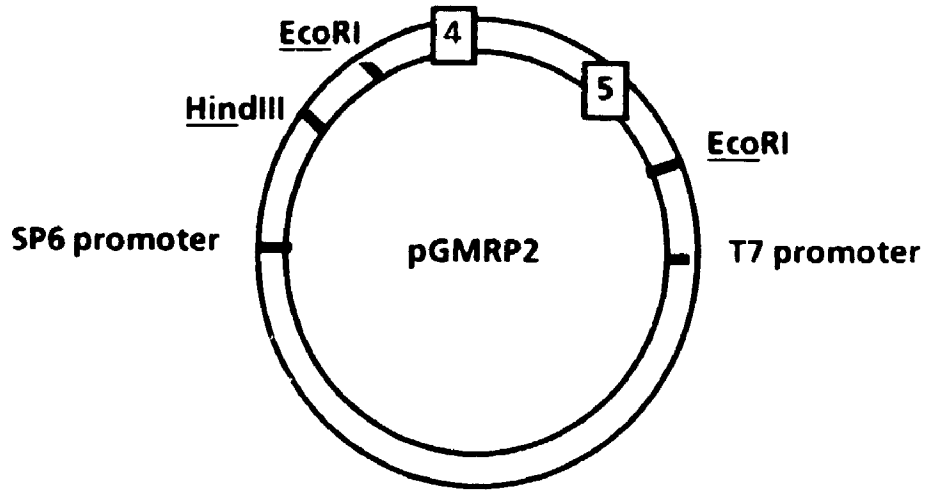
(Edwards et al., 1987). The results presented in 5.03 and 5.04 agree with the hypothesis that the mechanism responsible for the absence of MRP/PLF mRNA or protein in MEF cells is operating at a post-transcriptional rather than a transcriptional level. Mechanisms operating at a post-transcriptional level could involve rapid degradation of the newly synthesized RNA transcript, aberrant or incomplete processing of the transcript, or an inability to transport the processed message out of the nucleus. One way to look at all of these possibilities at once is to synthesize antisense RNA and hybridize it to transcripts isolated from both the nucleus and the cytoplasm. If the transcript is synthesized but then either not processed or not transported, a build up of transcripts would be observed in the nuclear fraction. Two different probes were used for these experiments. pGMRP2 is shown in Figure 21A. It was constructed by inserting the EcoRI fragment of p1.9Eco, which contains the final 2 exons of mrp/plf1, into the EcoRI site of pGEM3. Antisense RNA transcripts of the inserted MRP/PLF fragment were synthesized by initially cleaving the vector with HindIII and then using T7 RNA polymerase. It was felt that a probe consisting of only 2 exons would give cleaner results than a cDNA probe consisting of 5 exons. However, if MRP/PLF transcripts were terminated prematurely, they would not be observed with this probe so the pPH2GEM3 plasmid was constructed. pPH2GEM3 is composed of MRP/PLF cDNA from the PstI to HindII sites inserted in the

Figure 21 Cartoon of pGMRP2 and pPH2GEM3

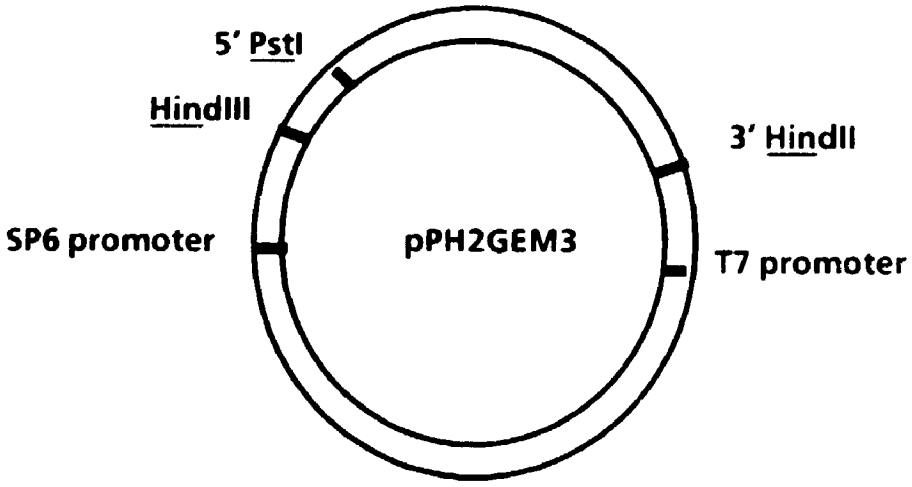
A: The 1.9-Kbp EcoRI fragment of mrp/plf1, which encompasses the final 2 exons and surrounding sequences, was inserted into the EcoRI site of pGEM3 to yield pGMRP2. It was oriented so the antisense transcripts can be generated by the use of the T7 promoter.

B: A full-length MRP/PLF cDNA was inserted into the PstI and HindII sites of pGEM3 to yield pPH2GEM3. It was oriented so the 5' end is at the PstI site and the 3' end is at the HindII site. Antisense transcripts can be generated by the use of the T7 promoter and sense transcripts can be generated by the use of the SP6 promoter.

A


EcoRI
4
5
HindIII
EcoRI
SP6 promoter
pGMRP2
T7 promoter

B


5' PstI
HindIII
3' HindII
SP6 promoter
pPH2GEM3
T7 promoter

pGEM3 vector and is shown in Figure 21B. Antisense MRP/PLF transcripts were synthesized by T7 RNA polymerase after pPH2GEM3 had been cleaved with HindIII. These probes were annealed to RNA from the nucleus or cytoplasm of both MEF and 3T3 cells as described in 2.47. Figures 22a and 22b show the results of probings with the antisense RNA from pPH2GEM3 and pGMRP2 respectively. When the lanes containing RNA isolated from 3T3 cells are examined, it is evident that the results with the probe containing only 2 exons are much cleaner than those with the probe containing 5 exons. This is because fewer intermediate species bind. There is no evidence of hybridizing MRP/PLF RNA in either the cytoplasm or nucleus of MEF cells while hybridizing RNA is observed in both the nuclear and cytoplasmic fraction of 3T3 cells. This result was expected for the MEF cytoplasmic RNA fraction but since MRP/PLF was transcibed in nuclear run-on experiments, it had been anticipated that transcripts would be observed in the MEF nuclear fraction. To confirm these results, the experiments were repeated on another preparation of MEF nuclear RNA and a 2AR/osteopontin probe was included as a control for a message known to be expressed in MEFs. Again, RNA hybridizing to MRP/PLF was not observed in the MEF nuclear fraction, while the 2AR probe confirmed that the RNA was intact (Figure 22c). The background for the 2AR mapping experiment is high because the same conditions were used for this probe as were used for the MRP/PLF probe. In general, it is necessary to work

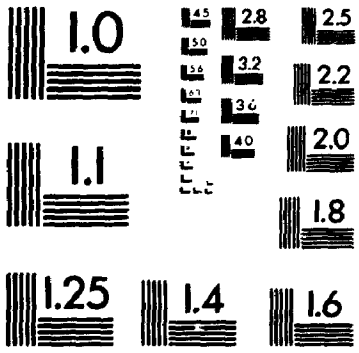


Figure 22 RNA Mapping MRP/PLF Transcripts in MEF and 3T3 Cells

High specific activity antisense transcripts were generated from pGMRP2 and pPH2GEM3 and annealed overnight at 50°C to RNA isolated from MEF nucleus, MEF cytoplasm, 3T3 nucleus or 3T3 cytoplasm. The samples were digested with RNase A and RNase T1. RNase-resistant fragments were ethanol precipitated, resuspended and run on an 8% acrylamide-7M urea sequencing gel. A 2AR cDNA probe was used to check the integrity of the MEF nuclear RNA.

Panels: a, RNA as indicated annealed with antisense pPH2GEM3 (MRP/PLF cDNA); b, RNA as indicated annealed with antisense pGMRP2 (mrp/plf1 final 2 exons); c, RNA as indicated annealed with antisense 2AR/osteopontin

out conditions for each probe in order to minimize background.

5.06 Discussion

The choice of restriction enzymes and probe in the experiments described in 5.03 enabled almost 7 Kbp of the region surrounding the MRP/PLF promoter to be inspected for DNase I hypersensitive sites. Only one such site was identified and it appears to be within 100 or 200 bases of the promoter. DNase I hypersensitive sites are free of nucleosomes and as such likely represent binding sites for proteins and enzymes involved in the regulation of transcription or the transcription process itself. The fact that this site is in the vicinity of the promoter suggests that it probably is significant in the function of this promoter. A hypothesis has been put forward that the presence of a DNase I hypersensitive site near the 5' end of a gene is necessary but not sufficient to allow transcription of that gene by RNA polymerase II (Elgin, 1981). If one accepts this hypothesis then it would appear that the chromatin structure of MEFs is suitable for MRP/PLF gene transcription. This is in agreement with the nuclear run-on experiments which suggested that MRP/PLF is transcribed to an equal extent in both MEF and 3T3 cells (Edwards et al., 1987).

The DNase I experiments indirectly reveal some other interesting information about the MRP/PLF multi-gene

family. Only one PstI hybridizing band was observed while two PvuII hybridizing bands were seen. This suggests that the MRP/PLF genes are similar until the upstream PstI site and diverge somewhere beyond this to generate the 2 PvuII fragments. It is not known how great this divergence is and it could of course just represent a point mutation in the genes so that one of them now has an extra PvuII site. Due to the lack of a probe specific for each gene, it is impossible to unequivocally assign the hypersensitive sites to a specific gene. The fact that a single hypersensitive site is observed after a PstI digestion and that the assignments of the DNase I sensitive sites after a PvuII digestion also map to this site strongly suggest that this same site is present in all the MRP/PLF genes that exhibit DNase hypersensitivity. This would imply that gene structures do not prevent transcription of MRP/PLF genes in MEF cells. Of course it could be argued that there is some region elsewhere in the genes which influences their transcriptional activity. Since most nuclease hypersensitive sites are found within 1000-bp flanking the 5' ends of transcriptionally active or inducible genes, this seems unlikely (Elgin, 1981).

The results obtained from the methylation experiments described in 5.04 are difficult to interpret. Firstly, even though all of the digestions were followed with lambda DNA and 10 units of enzyme was used per microgram of DNA, this still apparently was not sufficient to allow the HpaII

reactions to go to completion. The MspI digestions did not show this variation and always appeared as shown in 20a. Secondly, as seen by comparing the patterns in 20a and 20b, there are differences arising depending on the batch of HpaII enzyme used. Probably HpaII is exhibiting site preference, a phenomenon which occurs with NarI and NaeI restriction enzymes on pBR322 DNA. It can be concluded that in all the cell lines looked at, the MRP/PLF genes are highly methylated. It should be remembered that the use of HpaII/MspI is only allowing one to examine a small proportion of the possible methylated cytosine residues and just because this pair of enzymes has not picked up a difference between the MEF and 3T3 cells does not mean that one does not exist. This is exemplified by noting that there are 8 CG pairs present in the promoter region of mrp/plf1, but only 1 or 12% of these sites was looked at with MspI/HpaII digestions. Thirdly, more bands are lighting up with the probe than can be accounted for by the MspI/HpaII sites in mrp/plf1. This is likely due to the probe hybridizing to several different MRP/PLF genes. For instance, Table 8 shows that PLF42 and mrp/plf1 both have a C at position 29 while PLF149 has a T at this position. This destroys a HpaII/MspI site. There is also the possibility that methylation differences arise from cell to cell and therefore an all or none methylated site may not be seen. When one is observing a population, one is really looking at the average methylation status of a particular site.

It should be noted that the blots in Figure 20a and 20b also prove that the reason MRP/PLF is expressed in some cell lines is not a result of gene amplification in the expressing cell lines. Equal amounts of DNA were loaded and with the exception of the band already discussed, a given band appears to be approximately the same intensity in all the cell lines looked at.

The inability to detect MRP/PLF hybridizing transcripts in the MEF nuclear fraction indicates that incomplete processing or transport of the transcripts is unlikely to account for the lack of MRP/PLF expression in the MEFs. If one combines these results with the nuclear run-on data, (Edwards et al., 1987), a possible interpretation is that the MRP/PLF transcript is extremely unstable in the MEFs. mrp/plf1 does not have a particularly AU-rich sequence in its 3' untranslated region which has been proposed to be a site recognized by a specific endonuclease in some transiently expressed genes (Shaw and Kamen, 1986). It is therefore likely that a different mechanism is regulating MRP/PLF expression. There are a number of additional experiments which should be performed using nuclear run-on technology. The nuclear run-on experiments were probed with a full length double-stranded cDNA probe. The pPH2GEM3 vector allows the generation of antisense RNA transcripts from the T7 promoter and sense RNA transcripts from the SP6 promoter. The use of both of these probes would rule out the possibility that the transcription seen

was from a promoter in the opposite orientation to the MRP/PLF promoter. In addition, the mapping of mrp/plf1 in chapter 3 will allow the generation of probes directed toward either the 3' or 5' end of the gene so that it can be determined whether the entire gene is transcribed or only the 5' region. It is possible that there is transcriptional arrest within one of the exons. This control mechanism has recently been described as occurring in the c-myc gene in HL60 cells treated with DMSO or retinoic acid (Eick and Bornkamm, 1986., Bentley and Groudine, 1986). pGMRP2 will prove useful as a probe for the 3' end of the transcript since as shown in Figure 22b it gives clean results when incubated with total RNA.

One would perhaps have expected to see short transcripts in the MEF nuclear RNA if premature termination is occurring. Like the case presented here, Eick and Bornkamm (1986), were unable to detect c-myc transcripts after premature termination. The MRP/PLF transcripts, whether they are premature or full length must be extremely unstable, degrading either as soon as they are made or during the RNA isolation procedure. Perhaps if MEF cells were incubated with cycloheximide to inhibit protein synthesis, MRP/PLF transcripts might be observed. The results presented here suggest that the destabilization is occurring in the nucleus. If it were occurring in the cytoplasm one would have expected to see some transcripts in the nucleus. It has been suggested that dihydrofolate

reductase gene expression in cultured mouse cells is also regulated by stability of the transcripts in the nucleus which ultimately influences the amount of RNA available in the cytoplasm (Leys et al., 1984).

There are now several examples which implicate post-transcriptional mechanisms as being involved in the regulation of mRNA levels in addition to those mentioned above. During HL60 differentiation, the adenosine deaminase gene is transcribed at a comparable rate at both the 5' and 3' end of the gene yet its mRNA levels are repressed (Berkvens et al., 1987). Similarly, c-fos is transcribed in resting human monocytes but levels of c-fos mRNA were low or undetectable in these cells (Sariban et al., 1988). In the case of c-fos, it is proposed that sequences in the 3'-untranslated region of the mRNA are resposible for its stability.

The data presented here indicate that it is important to determine by nuclear run-on experiments which strand of the MRP/PLF genes is being transcribed and whether intragenic pausing is occurring. If the entire transcript is transcribed then destabilization of the message will be strongly implicated. One could then ask whether the instability is resulting from an RNase which is specifically found in the MEFs or whether there is a stabilizing protein in the 3T3s. One way this could be addressed is by transcribing MRP/PLF mRNA in vitro, adding it to the cellular extracts obtained from MEF and 3T3 cells, and

following the rate of its decay. Mixing experiments could also be done. A more complex version of this experiment would involve generating 3T3 lines with a stably integrated MRP/PLF gene carrying a marker so it can be distinguished from endogenous genes. The cells could be rendered permeable and then incubated in a solution containing cellular extracts from either MEF or 3T3 cells. This would be followed by examination of expression of the integrated gene. If a negative factor was present in the MEFs, there would probably be a decrease in expression of the integrated gene in cells incubated with cellular extracts from MEF rather than 3T3 cells. This factor could be further defined by fractionating the cellular extracts.

CAT assays would be a useful addition to the experiments described here to prove that MEF cells are capable of transcribing an MRP/PLF gene. When these experiments were tried using pSP<u>cat</u>, very little transcription was observed in the MEFs compared to the BNL cells. Not much weight should be placed on these results though because a lot of difficulty was encountered finding a 3T3 line which would express pSP<u>cat</u>. While it is known that MRP/PLF expression is very sensitive to culture conditions, this did not seem to be the problem because the 3T3 cell line which would transiently express MRP/PLF constructs, did so consistently. The problem also did not seem to arise from uptake or degradation of transfected DNA since with the exception of an NIH3T3 line, the pSV2<u>cat</u> control showed high activity in

all cell lines looked at. In order for these experiments to be meaningful, the MEFs from which the suitable 3T3 line was derived would have to be tested. Unfortunately, such a pair is unavailable in our laboratory.

Although the conclusions drawn in this thesis are that MRP/PLF is regulated post-transcriptionally in the MEFs, it is possible that the nuclear run-on experiments did not accurately represent the *in vivo* situation and MRP/PLF is not transcribed in MEFs. One could imagine that during the nuclear isolation procedure there was migration of a component normally sequestered in the cytoplasm to the nucleus which subsequently stimulated transcription. Perhaps even the chemicals themselves perturb the normal situation. The results presented in 5.03 and 5.04 have virtually ruled out the possibility of major structural changes in the MRP/PLF genes as MEFs become a 3T3 line so if the MRP/PLF genes are not transcribed it is probably because the MEFs have a negative regulatory protein or the 3T3s have some factor necessary for MRP/PLF transcription that the MEFs lack. In either case, the transcriptional activator/deactivator is not necessarily interacting directly with MRP/PLF genes but could instead be modifying another transcriptional factor to render it active/inactive. Regardless, the type of experiments described for looking at a factor involved in the stabilization/destabilization of transcripts could be performed. One could work out an *in vitro* transcription assay to see if nuclear extracts from both MEF and 3T3

cells can support MRP/PLF transcription. It would be of interest to see if extracts from different 3T3 lines showed variability as they did in the CAT assays or whether they would all support transcription. If MRP/PLF is under transcriptional regulation in the MEF and 3T3 cells then gel mobility shift assays could be used to approximate the region of the gene (presumably the promoter) that binds the factors. This region could be further defined using DNase footprinting assays.

In conclusion, it appears as if the change in MRP/PLF expression occurring as the MEFs are passaged to become a 3T3 line results from a change in a regulatory protein and not from a dramatic change in the chromatin structure or methylation status of the gene. At present it is not known whether this regulatory protein is involved in the initiation of transcription of the stabilization of the transcripts. A change in the expression of a regulatory protein would be consistent with the theory that the immortalization of cell lines results from a mutation. Mutation of this regulatory protein might not only permit the expression of MRP/PLF but also the expression of proteins directly involved in the immortalization process.

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