Tissue specific expression and regulation of the Mitogen Regulated Protein genes

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Tissue specific expression and regulation of the
Mitogen Regulated Protein genes

by

John Tollen Fassett

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
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Major: Molecular, Cellular and Developmental Biology

Major Professor: Marit Nilsen-Hamilton

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# TABLE OF CONTENTS

## CHAPTER 1: INTRODUCTION

- Introduction .................................................................................................................. 1
- Dissertation organization .............................................................................................. 7
- References ...................................................................................................................... 7

## CHAPTER 2: LITERATURE REVIEW

- Identification of MRP/PLF, a member of the prolactin/growth hormone family ............. 12
- Tissue specific expression and functions of MRP/PLFs .................................................. 15
- The regulation of MRP/PLF expression in cultured cells .............................................. 18
- Post-transcriptional regulation of mrp/plf transcripts .................................................... 22
- Regulation of mrp/plf expression in the placenta .......................................................... 24
- Post- translational regulation of MRP/PLF .................................................................... 33
- References ...................................................................................................................... 36

## CHAPTER 3: MRP4, A NEW MITOGEN-REGULATED PROTEIN/PROLIFERIN GENE; UNIQUE IN THIS GENE FAMILY FOR ITS EXPRESSION IN THE ADULT MOUSE TAIL AND EAR

- ABSTRACT ...................................................................................................................... 49
- INTRODUCTION .............................................................................................................. 60
- MATERIALS AND METHODS ....................................................................................... 52
- RESULTS ......................................................................................................................... 60
A new mrp/plf mRNA that is expressed in the midgestational placenta .................................................................60

Cloning of mrp4 ....................................................................................................................................................61

Mrp4 expression in the placenta ..............................................................................................................................61

MRP4 is glycosylated differently from PLF1 ...........................................................................................................62

Differential cell and tissue-specific expression of mrp/plf genes ...........................................................................64

Identification of the mrp4 promoter ..........................................................................................................................65

Localization of MRP4 in the hair follicles of the mouse tail ..................................................................................66

DISCUSSION ..........................................................................................................................................................67

ACKNOWLEDGMENT ........................................................................................................................................71

REFERENCES ......................................................................................................................................................71

FIGURES ..............................................................................................................................................................78

CHAPTER 4: MRP3, A MITOGEN-REGULATED PROTEIN/PROLIFERIN GENE EXPRESSED IN WOUND HEALING AND HAIR FOLLICLES ..........88

ABSTRACT .............................................................................................................................................................88

INTRODUCTION ...................................................................................................................................................89

RESULTS ..............................................................................................................................................................92

Mrp/plf Genes Are Expressed in Regenerating Epidermis .........................................................................................92

Mrp3 Is the Major Mrp/plf Gene Expressed During Wound Healing ........................................................................93

Keratinocyte Regulation of Mrp/plf Expression in Response to Growth Factors .........................................................93

Mrp3 Transgene Expression in Response to Wound Healing ....................................................................................94

Presence of MRP/PLFs in the Hair Follicle ............................................................................................................95
Upstream sequences repress functional expression and produce transcripts which are poorly spliced. ......................................................... 147

Discussion ........................................................................................................ 148

References ........................................................................................................ 155

Figures .............................................................................................................. 164

CHAPTER 6: CONCLUSION .............................................................................. 176
Tissue and temporal specific expression of different mrp/plf genes .... 176
Extra-placental expression of mrp/plf genes.................................................. 177
MRP/PLF expression in the placenta .............................................................. 180
Potential mechanisms of tissue specific variation in mrp/plf expression .......... 183
Analysis of the mrp3 promoter in transgenic mice .................................... 188
Finding endogenous mrp/plf enhancers ....................................................... 189
Tissue specific expression with a ubiquitous enhancer ......................... 190
Mrp3 upstream sequences effect splicing of the transgene ................. 192
References ........................................................................................................ 200

APPENDIX: IDENTIFICATION OF PROMOTERS FOR THE MRP/PLF GENES ...................................................................................... 209
Materials/Methods .......................................................................................... 209
Identification of the mrp4 and plf1 promoter ............................................. 209
Chapter 1: Introduction

Introduction

Communication between cells or tissues is essential to the viability of multicellular organisms. One mechanism by which cells communicate is through the secretion of growth factors or hormones, which travel through body fluids and bind to receptors on the surface of target cells. The binding of growth factors to specific cell surface receptors induces a cascade of intracellular events that often results in an altered profile of gene expression in the target cell. The products of these newly induced genes may then effect further changes within the target cell, including cell growth, differentiation, migration, or even programmed cell death. Often, the binding of a hormone or growth factor to its cognate receptor will result in increased synthesis and secretion of other growth factors, which, upon release, can induce even further physiological effects beyond those immediate responses elicited by the original growth factor. Growth hormone, for example, expressed in the anterior pituitary gland during childhood, binds to receptors on the liver and stimulates expression of insulin like growth factor I (IGF 1). IGF 1 then binds to IGF receptors displayed on bone cells to promote skeletal growth. A defect in the ability to produce growth hormone leads to reduced levels of IGF, which in turn slows skeletal growth, finally resulting in dwarfism (1). These types of interactions between different tissues that are coordinated by growth factors, are essential to normal growth, development, and reproduction of multicellular organisms. Therefore, it is important to unravel the
complex mechanisms by which growth factor signalling is regulated in order to understand how communication between cells or tissues relates to development and disease.

A major component of growth factor regulation is the tissue and temporal, specific expression of the growth factor. This control is often achieved through the differential expression of transcription factors that regulate expression of the certain genes encoding growth factors. The subset of transcription factors expressed in a cell and their activities are determined by the cell type and the external stimuli the cell receives. Transcriptional activation of a gene will occur through complex interactions between cell specific and ubiquitous transcription factors that bind to the promoter and surrounding sequences of the gene to promote RNA polymerase activity. In this manner, a cell type may be predisposed, based upon the cell specific transcription factors it contains, to transcribe and express a particular growth factor gene when induced by the correct stimuli.

The overall purpose of the research presented here is to investigate the tissue specific regulation of a subset of genes in the Growth Hormone/Prolactin (GH/PRL) family called Mitogen Regulated Protein (MRP; also known as Proliferin (Pif) and collectively as mrp/pifs. (Linzer and Nathans, 1984; Nilsen-Hamilton et al., 1980; Parfett et al., 1985). Previous to the reports included in this thesis, the only identified source of mrp/pif expression In vivo, were the trophoblastic giant cells of the placenta (Lee et al., 1988). These cells lie at the
interface between the fetal placenta and maternal decidua, where they also produce several other GH/PRL family members (Soares et al., 1998). During mid-gestation, giant cells secrete high levels of MRP/PLF (Lee et al., 1988; Linzer et al., 1985; Nilsen-Hamilton et al., 1988), where it is believed to act as both a placental angiogenesis factor (Jackson et al., 1994; Volpert et al., 1996) and a uterine growth factor (Fang et al., 1999; Nelson et al., 1995). MRP/PLF is also released into the maternal and fetal circulation. There is evidence that PLF1 binds to the mammary gland (JN ; Thesis) and the fetal heart (Jackson and Linzer, 1997). Thus, MRP/PLF is probably a fetal growth factor involved in coordinating maternal and fetal development during pregnancy.

Unlike other members of the GH/PRL family, which were originally identified in the pituitary or placenta, MRP/PLFs were originally identified as a family of glycoproteins secreted by growth factor stimulated NIH 3T3 cells, which are a cell type derived from fetal mesenchymal cells (Nilsen-Hamilton et al., 1980). Further investigations have confirmed that mrp/plf s are secondary response genes upregulated by growth promoting agents in 3T3 cells and some other immortalized cell lines. (Chiang and Nilsen-Hamilton, 1986; Gil-Torregrosa et al., 1994; Linzer D.I.H., (1984); Linzer and Wilder, 1987; Parfett et al., 1985). Southern blot analysis has revealed that there are between 4 and 6 mrp/plf genes in the mouse genome (Jackson-Grusby et al., 1988) and three separate but highly homologous mrp/plf promoters (96-98% identical) have been cloned (Connor et al., 1989; Linzer and Mordacq, 1987). Three different mrp/plf cDNAs
have been identified as well (Plf1, Plf2, and mrp3), which retain 96-98% homology, but only one of these (mrp3) has been linked to a cloned promoter.(Connor et al., 1989; Linzer and Mordacq, 1987; Wilder and Linzer, 1986). Mrp/plf promoters fused to reporter genes are regulated in transfected cells in response to serum or growth factors (Connor et al., 1989; Groskopf and Linzer, 1994; Linzer and Mordacq, 1987; Mohideen et al., 1999). However, despite extremely high homology amongst the three cloned mrp/plf promoters (98%) these promoters behave differently in response to various stimuli (Conner, 1986; Linzer and Mordacq, 1987; Mohideen, 1995; Mohideen et al., 1999). Furthermore, previous reports have shown that 3T3 cells only produce plf1 mRNA (Mohideen, 1995), while the placenta produces at least three different mrp/plf mRNAs (Mohideen, 1995; Wilder and Linzer, 1986). These observations suggested that individual endogenous mrp/plf genes are regulated differently from one another.

The observations described here suggest that elements within the mrp/plf promoters or surrounding sequences regulate tissue specific mrp/plf expression. To gain a better understanding of tissue specific regulation of mrp/plf gene expression the following questions were considered. 1) Which of the three to six possible mrp/plf genes are expressed in the giant cells; 2) What is the significance of multiplicity of highly similar mrp/plf genes? 3) Are different forms of mrp/plf expressed in particular temporal or tissue specific patterns, as is found with other genes for which there are multiple isoforms? 4) How is mrp/plf
expression directed specifically to the trophoblastic giant cells. The research described in this manuscript was designed to answer these questions.

To answer the questions posed in the previous paragraph, we have designed a quantitative method to determine the relative levels of different mrp/plf RNA species in a sample. We have used this assay to identify the major placental species and have demonstrated that different mrp/plf isoforms are temporally regulated during gestation. Furthermore, this assay was used to identify a fourth previously undescribed mrp/plf species (mrp4) which I cloned and characterized in terms of its tissue specific and temporal expression pattern. I also demonstrate that mrp/plf expression is not limited to the placenta, and that despite 97% sequence identity in the proximal promoter sequences, different forms of mrp/plf are indeed expressed in a tissue specific fashion in the adult mouse. Different patterns of mrp/plf expression were found in a number of adult epithelial tissues including the lining of the alimentary tract, the hair follicles, the tail and ear skin, and in healing skin wounds. Mrp/plf expression during different stages of wound healing and hair follicle growth was further investigated and was shown to be dynamically regulated during the healing process and through the hair follicle cycle respectively. Furthermore, primary cultures of mouse keratinocytes showed regulated expression of mrp/plfs in response to growth factors that are expressed during wound healing.

To better understand the mechanisms by which mrp/plf expression is regulated in vivo, mrp3 promoter/lacZ constructs were analyzed in cell culture
and in transgenic mice. It was demonstrated that 1450 b.p. mrp3 5' flanking sequence and 3000 b.p. of 3' sequences were sufficient to express detectable levels of β-galactosidase in transient transfection of giant cells or fibroblasts, but were unable to direct detectable reporter gene expression in transgenic embryos. A possible explanation for these results is that mrp3 promoter activity is inhibited when integrated into chromatin. To determine if a strong enhancer could overcome the inhibition by chromatin, the CMV immediate early enhancer was added to the 5' end of the transgene. The CMV/mrp3/lacZ transgene was able to direct expression of β-galactosidase activity in mid-gestational embryos and in the adult mouse, which paralleled expression of the endogenous mrp3. We further demonstrated that removal of 5' or 3' mrp3 sequences results in a higher level of non-specific expression more similar to the described expression pattern of CMV enhancer/promoter transgenes. This suggests that mrp/plf sequences in the transgene are able to direct tissue specific expression, but additional sequences are necessary to overcome inhibitory chromatin structure. Of significance is the finding that mrp3 sequences can direct tissue specific expression in the presence of an enhancer that is nearly ubiquitously active. The mechanism of tissue specific expression in the presence of a enhancer which is ubiquitously active is further investigated in this thesis.
Dissertation organization

The author did all of the research described in this thesis. The thesis is divided into five chapters. Chapter 1 is a general introduction to the project. Chapter 2 is literature review, in which background information regarding MRP/PLF is discussed. Chapters 3 through 5 are in the form of journal papers. In chapter 3 is described the analysis of which mrp/plf forms are expressed in the placenta and the identification and characterization of mrp4, a newly discovered mrp/plf family member with a distinct tissue and temporal specific regulation. Chapter 4 describes an investigation of the regulation of mrp/plf expression during the hair follicle cycle and in healing skin wounds of the mouse. In chapter 5 is described the investigation of mrp3 promoter regulation using transgenic mice and cultured cells. Chapter 6 is a discussion and interpretation of the results obtained in chapters 3 through 5 based on current understanding of the field. This chapter remarks on the significance of the findings presented and suggests further directions for this research. An appendix is also provided which includes other data obtained by the author that supports the thesis but is not included in papers to be published.

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*Biochimica et Biophysica Acta, 1009*, 75-82.


CHAPTER 2: LITERATURE REVIEW

Identification of MRP/PLF, a member of the prolactin/growth hormone family

Mitogen Regulated Protein (MRP) was originally identified by Marit Nilsen-Hamilton as a 34 kd glycoprotein secreted by immortalized mouse embryo fibroblasts (3T3 cells) in response to mitogen stimulation (Nilsen-Hamilton et al., 1980). The cDNA was later independently isolated from a library containing cDNAs of NIH 3T3 cell RNAs upregulated in response to serum stimulation. These investigators called this mRNA proliferin (Linzer and Nathans, 1984). Because MRP and proliferin are the same (Nilsen-Hamilton et al., 1987), the terms mrp/plf, and MRP/PLF will be used as a general reference to the genes or mRNAs and the proteins respectively. Specific genes and their products will be identified by their individual names such as mrp3, plf1, or plf2.

When the cDNA of plf1 was sequenced, it was found to have 55% nucleotide sequence identity with the coding region of bovine prolactin. Similarly, the open reading frame predicted a protein of 234 amino acids that had 22% homology to bovine growth hormone and 37% homology to bovine prolactin (Linzer and Nathans, 1984). The predicted structure of MRP/PLF is also similar to GH/PRL proteins, containing conserved locations of cysteines that are proposed to determine GH and PRL secondary structure (Dai et al., 1996). The gene
structure of mrp/plf is similar to other members of the gh/prl family; being composed of four introns and five exons (Connor et al., 1989). An important difference however, is that the MRP/PLFs, in contrast to GH and PRL, are always found glycosylated (Nilsen-Hamilton et al., 1981; Nilsen-Hamilton et al., 1980; Lee and Nathans, 1987). This post-translational modification has a significance that may be related to the functions of MRP/PLFs and will be discussed later. Because MRP/PLFs resemble GH/PRL in sequence and structure, yet do not bind to the GH/PRL receptors (Lee and Nathans, 1988), MRP/PLFs have been classified as a "non-classical" member of the gh/prl gene family.

The mrp/plf genes are located in a cluster on chromosome 13 of the mouse. This chromosome also harbors mouse placental lactogens I and II, proliferin related protein, and genes for at least 5 different prolactin-like proteins (Linzer and Fisher, 1999; Soares et al., 1998). These genes are believed to be a result of duplications of the prolactin gene, which is also located on chromosome 13 (Jackson-Grusby et al., 1988). All of these genes, including mrp/plf, are expressed in the placenta or uterus and for many of these genes, these tissues are the only known location of their expression (Faria et al., 1991; Linzer et al., 1985; Muller et al., 1998a; Muller et al., 1998b; Nilsen-Hamilton et al., 1991; Toft and Linzer, 1999; Yamaguchi et al., 1994) and (Linzer and Fisher, 1999; Soares et al., 1998) for review. At this time, a human MRP/PLF has not been identified.
However, humans have independently evolved placental specific members of the GH/PRL family from the growth hormone gene rather than the prolactin gene (Owerbach et al., 1980), and these hormones may perform some of the functions of the MRP/PLF.

Like other members of the GH/PRL family, gene duplication of mrp/plf has occurred. There are an estimated 4-6 mrp/plf genes based on Southern blot analysis (Jackson-Grusby et al., 1988), and three different cDNAs (mrp3, plf1 and plf2) have been identified. The plf1 cDNA was isolated from 3T3 cells (Linzer and Nathans, 1983; Linzer and Nathans, 1984), The plf2 cDNA was isolated from Balb/c mouse placentae (Wilder and Linzer, 1986), and the mrp3 cDNA is predicted from the sequence of the mrp3 gene, based on the exon/intron boundaries (Connor et al., 1989). Another form of mrp/plf mRNA exists as well in Ehrlich ascites carcinoma cells. This form is a splice variant of plf1 (Torregrosa B.G., 1994). The different mrp/plf cDNAs have 97-98 % nucleic acid identity, as do the first 600 bp of the three mrp/plf promoters that have been cloned (Connor et al., 1989; Linzer and Mordacq, 1987). Only the mrp3 promoter has been linked to a specific cDNA (mrp3) (Connor et al., 1989).

The significance of multiple similar genes is not known but it appears that despite few differences in sequence, the three mrp/plf promoters are regulated differently in transfected cells (Linzer and Mordacq, 1987; Mohideen et al., 1999). Linzer et al. (Linzer and Mordacq, 1987) reported that 1100 bp of the
promoters that he designated plf42 and plf149 were both active in transfected mouse L cells, but only the plf42 promoter showed induction in response to serum. Similarly, Mohideen et al. showed that in 3T3 cells, the mrp3 promoter was upregulated in response to fibroblast growth factor 2 (FGF2), while the plf42 and plf149 promoters were not (Mohideen et al., 1999). These differences in response to stimulation with various mitogens could reflect properties of the mrp/plf genes that allow tissue specific or temporal regulation of expression of particular mrp/plf species in response to various stimuli. Tissue specific or temporal activity of different mrp/plf promoters or genes in vivo, however, has not yet been reported.

Tissue specific expression and functions of MRP/PLFs

MRP/PLF was discovered in growing, immortalized mouse embryo fibroblast cells (3T3 cells) and can also be found in several other immortalized cell lines (Linzer D.I.H., (1984); Parfett et al., 1985) derived from other mouse tissues. But, it is not detectable in primary cultures of mouse embryo fibroblasts, the cultures from which 3T3 cells were derived (Parfett et al., 1985). A study to investigate where mrp/plf was expressed in vivo, a survey of adult and fetal tissues by Northern blot analysis revealed the placenta as the only tissue that contained detectable levels of mrp/plf mRNA (Linzer et al., 1985).

In the placenta, MRP/PLF protein and mRNA are localized specifically in the trophoblastic giant cells (Lee et al., 1988). Upon differentiation, trophoblastic
giant cells cease dividing, but continue to replicate their DNA, becoming very large polyploid cells that contain up to 1000 times the haploid amount of DNA (Barlow, 1972; Zybina, 1970). Giant cells are located in the outermost fetal layer of the placenta in direct contact with maternal tissue and are proposed to serve as a protective and selective barrier between the mother and the fetus (Hoffman L.H., 1993). From this location, giant cells produce MRP/PLFs at peak levels during midgestation (e10-11) at which time it is secreted to reach up to 5 ug/ml into the maternal bloodstream and 0.8 ug/ml into the amniotic fluid (Lee and Nathans, 1987). MRP/PLF is considered a non-classical member of the PRL/GH family in that it does not bind to the GH or PRL receptor (Lee and Nathans, 1988). This suggested that MRP/PLF functions through a different receptor and could have separate activities from classical members of the GH/PRL family. Indeed, Jackson et al. have shown that plf1 has angiogenic properties and can bind endothelial cells in the placenta, suggesting it is a placental angiogenesis factor (Jackson et al., 1994). This activity is mediated through the cation independent IGFII/mannose 6-phosphate receptor that is activated by mannose 6-phosphate residues on glycosyl groups of plf1 (Volpert et al., 1996). Interestingly, the mannose residues on circulating MRP/PLF in the maternal serum are not phosphorylated (Adnan Mubaidin and Yu Fang, unpublished results), so the mannose 6-phosphate activity of MRP is likely to be a very local effect upon only the placenta. However, Nelson et al. have shown
that MRP/PLF also binds to a receptor in the uterus that is different from the mannose-6 phosphate receptor. This receptor is temporally regulated with the highest expression occurring at the same time MRP/PLF is most strongly expressed (e11) (Nelson et al., 1995). Importantly, this report also shows that MRP/PLF stimulates DNA synthesis in primary uterine cell cultures, suggesting that MRP/PLF is a uterine growth factor. These results provide evidence that MRP is a fetal protein that probably plays a role in coordinating fetal/maternal interactions during pregnancy.(Fang et al., 1999)

Finally, there is another possible function of MRP/PLFs. That is, as an intracellular repressor of muscle cell differentiation. Wilder et al. showed that over expression of PLF1 in an immortalized myogenic cell line (23A2) can prevent muscle cell differentiation, but PLF2 does not have this effect (Wilder and Linzer, 1989). The expression of PLF1 in muscle cells apparently decreases the level of cARG transcription factor, which activates genes associated with myogenic differentiation (Muscat et al., 1991).

While it is now well established that members of the mrp/plf family are expressed strongly in the placenta, the levels and temporal regulation of individual mrp/plf forms in the placenta have not been thoroughly investigated. Linzer and colleagues have presented evidence using oligonucleotides specific for plf2 or plf1 are both of these genes are expressed in the placenta (Wilder and Linzer, 1986), and Mohideen, using an RT-PCR-restriction analysis assay, has
shown evidence for the presence of all three known cDNAs in the placenta (Mohideen, 1995). However, neither of these studies were quantitative, nor were they performed at multiple stages during the time course of *mrp/plf* expression. Because different MRP/PLFs may have different functions (Wilder and Linzer, 1989), and the promoters for the different *mrp/plf* genes appear to be regulated differently in vitro (Linzer and Mordacq, 1987; Mohideen et al., 1999), it would be of interest to accurately determine the expression patterns of different *mrp/plf* in vivo. This would further be of value if the particular promoters which have been identified (*mrp3, plf42, and plf149*) could be linked to tissue specific or temporally regulated mRNAs, as previous work investigating the regulation of these promoters could provide clues as to what factors may regulate the activities of the *mrp/plf* promoters in vivo.

**The regulation of MRP/PLF expression in cultured cells**

Studies regarding regulation of *mrp/plf* expression were initiated when it was found that secreted MRP/PLF levels increase substantially in response to mitogenic stimulation (Nilsen-Hamilton et al., 1980). These mitogens include serum, EGF, TGFα, PDGF, and bFGF (Linzer and Nathans, 1983; Nilsen-Hamilton et al., 1988; Nilsen-Hamilton et al., 1980). Nilsen-Hamilton and colleagues showed that, in quiescent 3T3 cells, 18 hours of treatment with EGF or TPA induced a 8 or 3 fold increase respectively in the amount of secreted metabolically-labeled MRP/PLF (Nilsen-Hamilton et al., 1988). In growing cells,
growth factors also stimulate a similar fold increase in MRP/PLF expression, however, the basal level of expression is nearly 10 fold higher than that of quiescent cells, so the overall difference between quiescent untreated cells and growing, growth factor treated cells can be as high as 70 fold. This data demonstrated that mrp/plf expression increases in response to growth factors and the overall level of expression is dramatically affected by the growth state of the cell.

Mrp/plf mRNA levels increase in response to mitogens (Nilsen-Hamilton et al., 1987) (Linzer and Nathans, 1984; Linzer and Wilder, 1987; Mohideen et al., 1999). The increase in mrp/plf expression in response to growth factors or serum correlates with entry into G1 of the cell cycle, and cells that have reached confluency express much lower levels of mrp/plf (Linzer and Wilder, 1987). Further characterization of the increase in mrp/plf mRNA showed that cycloheximide treatment during stimulation prevented the growth factor-induced increase in mrp/plf mRNA, suggesting that translation was necessary prior to upregulation of the message (Linzer and Wilder, 1987). Presumably, new transcription factors are being produced in response to mitogen stimulation, and these may be necessary to induce mrp/plf transcription. Analysis of the mrp/plf promoters for regulatory elements that may be involved in transcriptional regulation revealed a number of previously described transcriptional elements within the first 600 bp of the three separate promoters which have been
sequenced; these include a TATA box, AP2 site, GATA factor binding elements, AP1 sites, SPH elements, and a glucocorticoid response element. Deletion and mutation analysis of these promoters has revealed roles for a number of these elements in growth factor related activity. AP1 sites and three separate SPH sites located within -204 to -231 of the transcriptional start site are required and have a synergistic effect upon promoter activity in response to serum (Groskopf and Linzer, 1994; Mordacq and Linzer, 1989). A composite glucocorticoid binding site located within this region has also been investigated. This site is able to depress serum induced promoter activity in transfected L-cells in response to dexamethasome through an interaction with the glucocorticoid receptor (Groskopf and Linzer, 1994; Mordacq and Linzer, 1989). Interestingly, repression through this element requires interaction with Fos and Jun transcription factors, but interaction with Jun alone has a positive effect upon activity (Diamond et al., 1990). It has been suggested that the repression of mrp/plf promoters by glucocorticoids could be related to the decrease in placental mrp/plf expression during late gestation when higher levels of glucocorticoids accumulate (Mordacq and Linzer, 1989).

Like the cDNAs which have been identified so far, the sequences of the mrp/plf promoters that have been identified are ~ 97% homologous with one another. Mohideen et al used this homology, in combination with the results of studies of the ability of truncated mrp3 promoter fragments to respond to bFGF,
to identify a sequence involved in differences in bFGF responsiveness between the proximal 600 bps of the mrp3 and plf42 or plf149 promoters (Mohideen et al., 1999). They identified an element in the mrp3 promoter at -180 to -150 that differs by three base pairs from the site in plf42 and by 2 basepairs in plf149 and is able to induce a 2-4 fold increase in reporter gene expression in response to FGF. This element is termed the FRE, and can also be found in a number of other growth factor induced genes, particularly genes that encode secreted proteases (Mohideen et al., 1999). When placed upstream of a minimal TK promoter, the FRE confers FGF responsiveness upon that gene. Furthermore, proteins from nuclear extracts of 3T3 cells, placenta and embryo, but not liver, bind the FRE, suggesting that these proteins and/or this element may play a role in regulating a number of genes expressed during development (Hruska A.; Thesis, 1998). These investigations identified a few of the elements in the mrp/plf promoters that are probably involved in the transcriptional regulation of these genes, and they show that there are functional growth factor-inducible elements within the mrp/plf promoters. Furthermore, they show that subtle differences in the sequences of these elements can lead to different responses to external stimuli. It would therefore be of interest in this regard, to determine how the different mrp/plf promoters are regulated in vivo, so that the roles of the elements involved in mrp/plf promoter regulation could be given a physiological significance.
Post-transcriptional regulation of mrp/plf transcripts

Despite the apparent transcriptional regulation of promoter/reporter gene constructs in transfected cells, a puzzling piece of evidence exists, which suggests that the increase in endogenous mrp/plf expression in 3T3 cells in response to serum or growth factors is not due to an increase in transcription rate. Two separate groups have used nuclear run-off to measure transcription of mrp/plf genes in response to either serum or bFGF, but neither have detected an increase in the rate of transcription (Linzer and Wilder, 1987; Mohideen, 1995). Further analysis of the post-transcriptional regulation of the mrp/plf genes suggest that in unstimulated cells, the message is degraded in the nucleus, and it is suspected that an alteration in the ability to fully process the heterogenous RNA into mRNA is involved in the response to mitogens (Linzer and Wilder, 1987; Mohideen, 1995). A similar method of gene regulation has been attributed to the repression of mrp/plf expression in mouse embryo fibroblasts before they become immortalized, at which point mrp/plf expression begins (Edwards et al., 1987; Malyankar et al., 1994; Malyankar et al., 1996). Nuclear post-transcriptional regulation has also been described for a number of other genes including fibronectin, dihydrofolate reductase, interleukin-2 and hemoglobin genes (Chandler et al., 1994; Gerez et al., 1995; Kollias et al., 1996; Leys et al., 1984). In the case of fibronectin, activated ras mediates a down regulation of fibronectin at the level of nuclear processing of the primary transcript, while
Dexamethasone treatment increased fibronectin expression by stabilizing the primary transcript (Ehretsmann et al., 1995). Dihydrofolate reductase and interleukin-2 mRNA levels were both reduced due to inefficient processing of the primary transcripts in the absence of stimulation. The hemoglobin genes, which are developmentally regulated in erythroid cells by transcriptional control, also appear to be regulated at the post-transcriptional level as incompletely spliced transcripts of ε-globin are detectable in adult cell types, but the fully processed gene or protein is not detectable. Likewise, low levels of incompletely spliced β-globin genes are detectable in cells that express primarily the embryonic forms. The mechanism by which growth factors or developmental signals regulate nuclear processing or nuclear stability of transcripts in these or other cases is still not well understood, and could occur more often than what is reported, as unprocessed primary transcripts would likely be degraded in the nucleus and may not accumulate to easily detectable levels. The lack of correlation between mitogen regulated promoter activity in reporter gene assays and endogenous transcription also needs to be resolved if the true mechanism of Mrp/PIf regulation is to be understood. Because however, the promoter for each form of Mrp/PIf has not been identified, and because there are multiple Mrp/PIf genes, not distinguishable from one another by hybridization assays, evaluation of the precise mechanism of Mrp/PIf regulation may be complicated, particularly if the different Mrp/PIf genes are being differently regulated (eg. transcriptionally vs.
Regulation of *mrp/plf* expression in the placenta

While the promoters of *mrp/plf* genes have been investigated in cell culture, little is known about how these promoters are regulated in vivo. In the mouse, expression of *mrp/plf* has only been shown in the trophoblastic giant cells (TGCs). In these cells, which begin to form during the blastocyst stage, cell division has ceased, but DNA synthesis continues in a process referred to as endoreplication (Zybina, 1970). Endoreplication continues up to day 14 (Jollie, 1964). Interestingly, the period of highest level *mrp/plf* expression in the giant cells (e9-11) occurs at a time when TGCs are still forming and are found at high numbers (Barlow, 1972; Muntener and Hsu, 1977; Zybina, 1970). So like 3T3 cells, there may be a correlation between DNA synthesis and *mrp/plf* expression (Nilsen-Hamilton *et al.*, 1980). Therefore, some of the elements involved in growth factor induced increases in *mrp/plf* expression are likely to be the same in giant cells and 3T3 cells. However, because the giant cells are difficult to isolate and culture, the best way to identify elements of the *mrp/plf* promoters that are involved in giant cell regulation of *mrp/plf* expression is to produce transgenic mice using various *mrp/plf* promoter reporter gene constructs. This work has not been previously reported for the *mrp/plf* genes, but is carried out in the research described in this thesis.
A number of investigations have been carried out involving endogenous $mrp/plf$ expression in the placenta in various conditions that give insight into how these genes are regulated in vivo. The most significant finding was provided by Ma et. al who show that placentae genetically null for functional GATA-3 and especially GATA-2 transcription factor genes produce much less MRP/PLF than heterozygous placentae from the same mating (Ma et al., 1997). This, along with in vitro evidence that GATA factors regulate activity of the closely related Mouse Placental Lactogen I promoter (Ng et al., 1994) suggest that these transcription factors play an important role in $mrp/plf$ expression in vivo. Like the mPL I promoter, the $mrp/plf$ promoters contain a number of GATA sites through which regulation may occur. JunB, a member of the AP-1 complex, which has been demonstrated to be important transcriptional response to serum stimulation, has also been shown to be essential for correct placental development, placental $mrp/plf$ expression, and neovascularization (Schorpp-Kistner et al., 1999). Other studies have shown that cAMP treatment can increase the number of trophoblastic giant cells expressing $mrp/plf$ in vitro (Yamaguchi et al., 1995). This is believed to occur however, by increasing the number of cells differentiating into giant cells, and may not be a result of increased level of expression in each giant cell. Studies involving $mrp/plf$ expression in vitro have also been carried out on blastocyst outgrowths, in which giant cells were allowed to differentiate outside of the uterus. Here it was shown
that giant cells still progress through a similar order of expression of GH/PRL related genes including *mrp/plf*, suggesting that a major part of *mrp/plf* regulation is the differentiation state of the cell (Carney *et al.*, 1993). Another interesting aspect of *mrp/plf* regulation in giant cells is the down regulation of *mrp/plf* expression. In vivo, *mrp/plf* expression peaks between days 10 and 11 p.c. and then rapidly decreases. When removed from the uterus after day 11 however, and cultured in media, expression of MRP/PLF protein returns to high levels, suggesting that the uterus may play a role in down regulation of *mrp/plf* after e11 (Fang *et al.*, 1999). There is likely to be a uterine factor involved in MRP/PLF down-regulation, because co-culture in vitro of giant cells in placental minces with late stage uterus, but less so with early stage uterus, causes a specific decrease in secreted MRP/PLF similar to that seen in vivo. Also, late gestational uterine extracts specifically inhibit the production of MRP/PLF (Fang *et al.*, 1999).

Other insight into tissue specific activity of the *mrp/plf* promoter could be gleaned from results of experiments involving other genes expressed in the trophoblastic giant cells. The Mouse Placental Lactogen II promoter is expressed specifically in transgenic placentae if 2 kb of promoter sequence was used, but was inactive if only 1.3 kb was used. Transient transfection of a rat trophoblast cell line (RCHO-1) was then used to identify two previously undescribed AT-rich elements that are necessary for high-level expression in giant cells (Lin and
Linzer, 1998). These elements have not however, been identified in the mrp/plf promoters. Analysis of the placental lactogen I promoter was carried out by transient transfection in RCHO-1 cells and it was shown that expression from this promoter is dependent on GATA binding sites in the promoter and transcription is increased by exogenous expression of GATA factors 2 and 3 (Ng et al., 1994). Like the mPL I promoter, the Murine Adenosine Deaminase promoter (ADA) is also regulated by GATA factors. This gene is not a member of the GH/PRL family, but is expressed strongly in the placenta in all trophoblast lineages. Placental specific expression of ADA/cat transgenes depended on a 770 bp region located 5.4 kb from the transcriptional start site. This region contained multiple placental-specific footprints as well as footprints produced by non-placental tissues. Furthermore, this region contained a number of previously described elements involved in placenta specific expression, including GATA elements. In this case, mutation of two GATA elements within the 770 bp region in the ADA promoter decreased the level of expression but did not drastically effect tissue specificity of expression of reporter gene activity (Shi et al., 1997). Other elements were also found to be involved in placenta specific expression. Deletion of a region containing homology to the human placental lactogen enhancer completely diminished CAT reporter gene expression, even though it did not contain a footprint, as did a separate region that did produce a placenta specific footprint. This placenta-specific footprint contained a binding site for AP-
2 that is enriched in trophoblast lineages, and mutation of this site abolished placenta specific expression (Shi and Kellems, 1998). The *mrp/plf* promoters also contain an AP-2 site in the proximal promoter. Another region that produced footprints in both placenta and liver was also found to be necessary for placenta-specific expression (Shi *et al.*, 1997). These data suggest that GATA factors may play a role in placenta-specific expression, but other factors, both tissue specific and otherwise, are also involved, and like many enhancers and promoters, tissue specific expression is the result of an interplay between multiple elements.

One particularly well investigated example of how tissue specific gene expression may be mediated by surrounding sequences is the regulation of the pituitary specific human growth hormone gene (hGH) and it's closely related placenta specific family members. The GH gene is found in a cluster of five genes that are 93-96% similar in sequence identity in the proximal 5' flanking region and coding sequences (Miller and Eberhardt, 1983), but are expressed in distinct patterns. While hGH-N is only expressed at high levels in the anterior pituitary, hCS-A and B are strongly expressed only in the placenta, where the other two (hGh-V and hCS-L) are also expressed but at a weaker level. This system can therefore provide insight into how different tissue specific expression patterns are established even amongst genes that are highly similar.
Analysis of the promoter activity of hGH and hCS genes in transfected cells has revealed some differences in the proximal 5' or 3' flanking regions which effect pituitary or placental cell specific expression. For instance, the 0.5 kb 5' flanking regions of the hCS-A and hGH-N genes are both active in pituitary cell lines (Cattini and Eberhardt, 1987; Cattini et al., 1988; Nachtigal et al., 1989) and bind Pit-1 (Bodner 1987, Lefevre 1987) transcription factor, even though only hGH-N is expressed in the pituitary in vivo. The high level expression of pit-1 in the pituitary, and the ability of pit-1 to transactivate the hGH promoter in extracts of non-pituitary cells (Bodner and Karin, 1987) suggests that pituitary specific hGH promoter requires expression and activity of Pit-1. Furthermore, mutation of Pit-1 is a known cause of pituitary dwarfism, demonstrating the important contribution of Pit-1 to growth hormone expression (Frisch, 2000). The activity of the 0.5 kb hCS-A promoter in pituitary derived cells however, suggests that other sequences may be necessary for repression of hCS genes in the pituitary. In support of this, two specific “PSF” sequences within 1.7 to 2.1 kb 5' of each of the hCS genes can severely repress pituitary specific expression. These elements bind nuclear proteins from pituitary cell lines, but do not repress expression in placental cell lines or bind similar placental proteins (Nachtigal et al., 1993). The promoters for hCS-A and hGH-N appear to be in an “open” chromatin state in the pituitary (Nickel and Cattini, 1996), suggesting that the promoters are both accessible to trans-activating factors. It is therefore possible
that these hCS specific sequences may be involved in pituitary specific repression of placental GH genes (Nickel and Cattini) which is not mediated by chromatin mediated inaccessibility. The mechanism by which these repressors prevent expression of the hCS genes however, remains undetermined. There is also an enhancer described in the 3' end of the hCS-B gene which functions only in placental cell lines and may also contribute to placental specific activation of the hCS genes.(Jacquemin et al., 1994; Rogers et al., 1986). This enhancer binds specific proteins in the placenta, which are different from proteins bound in pituitary cells, where it also contributes to repression of hCS gene expression (Jiang and Eberhardt, 1997). Thus, while very proximal sequences (0.5 kb) are similar in sequence and activity, more distal sequences in the 5' and 3' flanking sequences and the proteins which bind there override the influence of the very proximal sequences to promote tissue specific transcription. Another level of regulation also exists in vivo however.

The experiments described above, carried out in for the most part in transiently transfected cell lines, may not adequately represent what occurs in vivo, particularly in the context of chromatin structure and development. For instance, even though the proximal 5' region (0.5 to 5kb) of the human growth hormone (hGH-N) promoter, is regulated by pit-1, in transfected pituitary cell lines (Bodner, 1988; Fox et al., 1990; Sittler and Reudelhuber, 1990), it is not expressed or is expressed at low and variable levels in the pituitaries of
transgenic mice (Bennani-Baiti et al., 1998) (Hammer RE, 1984; Jones et al., 1995). Also, a 15kb fragment containing 5kb of 5' and 7kb of 3' sequence of the hCS-A gene was poorly and variably expressed in the placentae of transgenics, suggesting that further elements are required for appropriate tissue specific expression of these genes. Indeed, it is now evident that high level, tissue specific and site-of-integration-independent expression of the hGH-N gene in pituitaries and placentae of transgenic mice depends on 4 DNAse hypersensitive sites (HS) between 14 and 40 kb 5' of the hGH-N gene (Bennani-Baiti et al., 1998; Jones et al., 1995). Two of these sites (HS I and II) are specific for the pituitary, while two other HS sites (HS iii and V) are found in both the placenta and the pituitary, and another HS site (HS IV) is found in only the placenta. The two most proximal pituitary specific HS sites at-14.6 and -15.4 (I and II) in their natural context or when isolated and linked to 0.5kb of the hGH promoter result in high level position independent transgene expression, but also allow slight ectopic expression of the transgene. Expression levels from the 0.5 kb hGH promoter were on average 1000 fold higher with addition of the HS I and II fragment than in it's absence. The two HS sites at -32.5 and -27.5 (HS III and V) also allow position independent transgene expression when attached as an 11.5 kb fragment to the 0.5 kb promoter, but expression levels are much lower than transgenes containing sites I and II. Again, ectopic expression is observed, this time consistently in the kidney. Here it is important to note that pit-1 is also
expressed in the kidney, suggesting that distal HS sites are necessary to allow pit-1 activity on the proximal 0.5kb promoter, but other sequences removed from this construct are required to repress this activity. Only the complete 40kb fragment containing all four HS sites allowed tissue specific and position independent transgene expression. Recently it has been demonstrated that the HS I and II sites in the hGH 5' region, which were specific for the pituitary, are dependent upon PIT-1 for activity (shewchuk 1999), demonstrating that the transcription factor PIT-1 plays an important role in the proximal promoter activity during transient transfection, as well as in distal sites which overcome inhibition by surrounding chromatin. Consistent with a role of LCRs in overcoming inhibitory chromatin structure is the finding that the hGH-N locus control region is a target for histone acetyl transferase activity in the pituitary that results in increased acetylation that spreads into the hGH-N gene, but does not extend into the placental genes (Elefant et al., 2000). Interestingly, transgenic mice containing the 40kb 5' region and all five genes of the hGH locus also express the human placental specific members of the locus at high levels in the placentae of transgenic mice.(Su et al., 2000), even though the placental members of the PRL/GH super family in rodents are derived from Prolactin. The complex regulation of the hGH gene cluster demonstrates the importance of both proximal promoter elements that can bind tissue specific transcriptional activators or repressors, and other possibly distant elements that are likely
involved in overcoming inhibitory chromatin structure.

Post-translational regulation of MRP/PLF

The MRP/PLFs are expressed from 3T3 cells as 34kd glycoproteins (Nilsen-Hamilton et al., 1980). In the placenta, the MRP/PLFs are found as 27, 34, and 38 kd forms, but the amino acid sequence predicts a 22 kd protein. These differences may be due to differential glycosylation states for specific MRP/PLFs, or to different levels of glycosylation on the same protein. Interestingly, the pattern of expression of these different molecular weight MRP/PLF species changes during gestation. While the higher molecular weight form (38kd) is expressed in early and midgestation, the 27kd form is expressed strongest on day 13 (Fang et al., 1999). The 27 kd form is only found in the placenta however, and not in the amniotic fluid or the maternal serum. This suggests that the 27 kd form is either not released into the bloodstream, is taken up rapidly by placental or decidual receptors, or is very unstable in the blood. The significance of different glycosylation states of MRP/PLFs has not been determined, but it could be envisioned that the level and type of glycosylation could correlate positively with the solubility or stability of MRP/PLF as it does for a number of proteins (Joannou, 1998; Melikian, 1996; Wang, 1996), including mPL1 and 2 (Kelly, 1975). Alternatively the glycosylation state could effect the interaction of MRP with a receptor. The glycosylation state could be especially relevant to interactions with the mannose-6 phosphate receptor, but at this time it is not
known which glycosylation site on MRP/PLF contains the mannose-6 phosphate residues that are involved in receptor binding.

In conclusion, the MRP/PLFs are growth factors and angiogenesis factors that are expressed by the mouse placenta and also expressed in immortalized mouse cell lines. The cDNAs of three out of four to six of the mrp/plf genes have been identified, and three separate but highly homologous mrp/plf promoters have been studied in transfected cells. The mrp/plf promoters are regulated differently in response to various stimuli, and transcriptional differences have been identified that are involved in mediating these differences. The physiological relevance of these differences in mrp/plf promoter regulation however, remain unknown. While it has been shown that the mrp/plfs are expressed by the trophoblastic giant cells and that expression peaks during mid-gestation, there are many questions remaining in regards to in vivo mrp/plf regulation. For example: What is the significance of multiple mrp/plf genes? Which mrp/plfs are the most prevalent in the placenta? Are they all expressed in the placenta at the same time, or might they be regulated differently from another in vivo, as the identified promoters are in vitro? Furthermore, what are the transcriptional elements that drive high levels of giant cell mrp/plf expression? This latter question cannot be answered until it is known which mrp/plfs are expressed in the placenta, so that the appropriate promoter may be analyzed.
Another question that remains is whether or not \( \text{mrp/plf} \) is really expressed only in the giant cells? This is an important question to ask if one is to form a hypothesis to explain tissue-specific expression of \( \text{mrp/plf} \). In the study by Linzer, in which the placenta was identified as the site of \( \text{mrp/plf} \) synthesis, the only other adult tissues examined were the liver, kidney, brain, and ovaries (Linzer et al., 1985). These organs do not contain a large number of proliferating cells. Because of the strong correlation between \( \text{mrp/plf} \) expression, mitogen stimulation, and DNA synthesis (Nilsen-Hamilton et al., 1980), it is possible that there are other sites in the adult mouse that contain cells proliferating in response to growth factors and where \( \text{mrp/plf} \) may be expressed.

Another question regarding tissue specific expression of the \( \text{mrp/plf} \) genes is based on the observation that the increases in endogenous \( \text{mrp/plf} \) expression levels in cultured cells do not appear to be transcriptionally regulated, but are regulated by a mechanism involved in processing of the primary transcript (Linzer and Wilder, 1987; Mohideen, 1995). Because this observation also applies to primary cultures of mouse embryo fibroblasts (Malyankar et al., 1994; Malyankar et al., 1996), it is possible that the \( \text{mrp/plf} \) promoters may be active in the intact mouse embryo as well, or even in the adult mouse, but that the message is not detected because of post-transcriptional degradation. Do trophoblastic giant cells increase \( \text{mrp/plf} \) expression by increasing the rate of transcription of the gene or is the mechanism of regulation post-transcriptional?
Perhaps the massive tissue specific increase in *mrp/plf* expression is a result of a combination of transcriptional and post-transcriptional regulation. This leaves open the question of what to expect when analyzing the activity of the *mrp/plf* promoters in vivo. If tissue specificity is solely under the control of the *mrp/plf* promoter, then tissue specific expression of a reporter gene under control of a transgenic *mrp/plf* promoter might be expected. If promoter activity is constitutive however, as the endogenous promoter for *plf*1 appears to be in 3T3 cells, and post transcriptional regulation occurs at the level of the primary transcript, then a reporter gene may not be similarly post transcriptionally regulated. In this case, transgene expression would appear non-specific, and further investigation into post-transcriptional processing of the *mrp3* gene would be necessary. Answering these questions regarding the expression patterns of multiple *mrp/plf* forms, and the activity of the *mrp/plf* promoters in vivo is crucial to our understanding of the function and regulation this gene family, and is therefore, the goal of the research described in this thesis.

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CHAPTER 3: MRP4, A NEW MITOGEN-REGULATED PROTEIN/PROLIFERIN GENE; UNIQUE IN THIS GENE FAMILY FOR ITS EXPRESSION IN THE ADULT MOUSE TAIL AND EAR

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ABSTRACT

Mitogen-regulated proteins (also known as proliferin; mrp/plf) are nonclassical members of the PRL/GH family. They are expressed at high levels during midgestation when they are thought to induce angiogenesis and uterine growth. There are between four and six mrp/plf genes, and three different cDNAs have been cloned. Here we identify a fourth mrp/plf gene (mrp4) that we have cloned and characterized. MRP4 is 91% identical in amino acid sequence with the other MRP/PLF proteins but is missing two glycosylation sites that are present in the other forms. Consistent with the loss of two of three glycosylation sites, the expressed form of MRP4 has a lower apparent molecular weight.

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compared to other MRP/PLFs. In vivo, *mrp4* is expressed in the placenta and the adult skin. Expression of *mrp4* mRNA peaks in the placenta on day 12. In the skin, *mrp4* expression is specific to the ears and tails of mice. Our results suggest that, as well as having growth and angiogenic effects during pregnancy, the MRP/PLFs may have functions in nonreproductive tissues. Unique amongst the members of the *mrp/plf* family for its expression in the hair follicles of the tail and ear, MRP4 is expected to have a singular role in the growth and development of these follicles.

**INTRODUCTION**

Proteins of the prolactin/growth hormone (PRL/GH) family play essential roles in growth and reproduction in mammals. Humans contain three placenta-specific variants of hGH (1) while rodents have evolved numerous placental-expressed descendents of prolactin (2). In the mouse, the placental members of the family include placental lactogens I and II (mPLs) (3-5), proliferin-related protein (PRP) (6); the more recently identified homologues of the rat prolactin-like proteins (PLP A,B, Ca, Cc, Cv, E through L, and d/t-PRP) (7-12), and the mitogen regulated proteins (proliferins; MRP/PLF) (13). MRP/PLFs are glycoproteins consisting of a polypeptide core of 21 kDa and appear by gel electrophoresis to be of average sizes varying from 27K to 38K. Like some other members of the PRL/GH family, the MRP/PLFs are synthesized in the placenta and their
synthesis is temporally regulated during gestation (6, 14). Expression of the mrp/plf genes in the placenta begins as early as E7, peaks at midgestation (E9-11), then declines through the remainder of gestation. In situ hybridization and immunohistochemistry have identified the trophoblastic giant cells as the site of MRP/PLF synthesis in the placenta, from which it is released into both the maternal bloodstream and the amniotic fluid (15). MRP/PLFs are believed to function during pregnancy as placental angiogenesis factors through their interaction with the mannose-6-phosphate receptor (16) and as uterine growth factors through another receptor that is specific for MRP/PLFs and does not recognize mannose-6-phosphate (17). PLF1 also has been described as having an intracellular role, in preventing muscle differentiation (18, 19).

The mouse genome contains between four and six mrp/plf genes (20) and previous to this report, three cDNAs had been cloned: plf1 from serum-stimulated 3T3 cells (21), plf2 from a Balb/c mouse placental cDNA library (22), and mrp3 from a Swiss mouse embryo fibroblast library (23). The three MRP/PLFs are ~98% identical in amino acid sequence and the variations in sequence do not disrupt any predicted structural motifs. Thus it is likely that all three previously identified MRP/PLFs have a similar structure. Each of the identified MRP/PLFs contain three N-glycosylation motifs within the sequence of the mature secreted protein, and all placental and 3T3 forms are glycosylated (13, 22, 24). Because MRP/PLF binds at least two different receptors during
gestation, only one of which recognizes mannose-6-phosphate, the level of glycosylation could be important in determining the functional interactions of the MRP/PLFs with their receptors in vivo (16, 17, 25).

Here we describe a fourth mrp/plf mRNA (mrp4) that we have cloned, sequenced and characterized. The mature protein produced from this newly discovered member of the mrp/plf gene family is 91% identical in amino acid sequence to the other members of the family. MRP4 lacks two of the three glycosylation sites found on the other MRP/PLFs, and when expressed, it appears to contain less carbohydrate than the other MRP/PLFs. Expression of mrp4 mRNA is consistent with the presence of MRP/PLF protein found in the placenta in the latter half of gestation and that has a lower apparent molecular weight than the plasma form of MRP/PLFs. MRP4 is also the only mrp/plf mRNA species detected in hair follicles of the tail and the ears of adult mice. These results suggest that, as well as functioning to stimulate uterine growth and placental vascularization during pregnancy, the MRP/PLFs also may have a specific function in adult hair follicles. Because mrp4 is the only member of the mrp/plf gene family expressed in the tail and the ear, this gene may play a singular role in these tissues.

MATERIALS AND METHODS

Animals and materials CF-1 and FVB mice, originally obtained from Charles River (Wilmington, MA), were bred and cared for at the Laboratory
Animal Facility at Iowa State University under a 12-h light/dark cycle. For pregnant animals, the day of the plug was counted as day 0. Animal care was provided by an animal caretaker and an attending veterinarian. This research was conducted in accordance with the standards set forth in the NIH guide for the care and use of laboratory animals. Animals were killed by cervical dislocation prior to removal of tissues for the described studies. Prior approval was obtained from the Iowa State University Committee on Animal Care for all procedures performed on the animals used in these studies.

Polyclonal anti-MRP rabbit sera and preimmune sera were prepared as described (26). pRSV-plf1 and pRSV-plf2 plasmids and a set of cosmids containing mrp/plf-hybridizing sequences were gifts from Jiandie Lin and Daniel Linzer (Northwestern University). The pBluescript II KS(+) plasmid (pBKSII+) was obtained from Stratagene (La Jolla, CA), and the pcDNA3 expression vector was obtained from Invitrogen (Carlsbad, CA). The full mrp3 gene extending from -1450 to 600 bp past the poly A site (including introns) was obtained from David Denhardt (Rutgers University) in four separate pieces. The full-length genomic clone from -1450 was reconstructed almost completely except for an approximately 230 basepair Accl-Pvull fragment of intron 1 and a 300 basepair EcoRI fragment of intron 3, which were removed during cloning. The reconstructed gene was placed downstream of the CMV (cytomegalovirus) enhancer (obtained from pcDNA3) to produce pCMV-MRP3. Tunicamycin was
from Sigma (St. Louis, MO). Dulbecco-Vogt's modified Eagles medium with high glucose (DMEG) was from Gibco BRL.

**RNA isolation and RT-PCR** Immediately after their removal, tissues were frozen under liquid nitrogen and stored at -70 C until use. Frozen tissue was pulverized in liquid nitrogen using a mortar and pestle, and RNA was isolated using Tri-Reagent (Gibco BRL) according to the manufacturer's instructions. For reverse transcription, 250 ng of total RNA was incubated with or without 40 units of Superscript II reverse transcriptase (Gibco BRL) in 5-μl reaction mixtures containing 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 112 ng poly dT primers, 400 μM each of the four dNTPs (dATP, dGTP, dCTP, dTTP), and 50 mM Tris·HCl (pH 8.3 at RT). The reaction mixtures were incubated at 42 C for 50 min, 50 C for 10 min, then 75 C for 15 min.

For PCR, the following primers were used; mrp/plf downstream exon 1 (DE1), 5'TAAGCCTGGGTAGGACTCTGC3' (+45 to +65), or (DE1B) 5'CTCTGCAGAGATGCTCCCTTC3' (+59 to +79) and mrp/plf upstream exon V (NUE5), 5'CATGATATTTTCAGAAGCAGAC3' (+778 to +795). Briefly, 0.5 μl of the reverse transcription reaction mix was added to a 25-μl volume containing 1 unit of Taq Polymerase, 20 pmoles of each primer, 200 μM of each of the four dNTPs, 1.5 mM MgCl₂, 50 mM KCl, and 10 mM Tris·HCl (pH 8.3). The thermal cycling conditions were as follows: 95 C for 2 min to denature, then 40 cycles at 95 C (30 sec), 66 C (1 min), and 72 C (1.5 min) with a final 3 min at 72 C. The PCR
products were resolved by electrophoresis through a 1.5% agarose gel and examined by ethidium bromide staining.

**Diagnostic restriction digestion to distinguish between the mrp/plf mRNA** The diagnostic RT-PCR assay for the mrp/plfs is based on minor differences in the cDNA sequences of these closely related gene products which result in the presence or absence of restriction sites within the cDNA that can be used to distinguish between the mrp/plf mRNAs (Fig. 1). For restriction digestion, one-fifth of the reaction mixture from the RT-PCR reaction was subjected to one additional round of PCR using DE1B and NUE5 primers and 62.5 μM of each of the four dNTPs, 0.9 mM MgCl$_2$ and 1 μCi $^{32}$P-αdCTP in a 25-μl volume. The sample was heated to 98°C for 4 min, 64°C for 1 min, then 72°C for 20 min. The radiolabelled cDNA was precipitated with 70% ethanol in the presence of 0.3 M sodium acetate with 10 μg yeast tRNA as a carrier. Digestion was carried out using 1 unit each of BsoFI (New England Biolabs, Beverly, MA) and BstXI at 55°C overnight, or 5 units of Ndel (Gibco BRL; Rockville, MD) at 37°C including 100 μg/ml bovine serum albumin (New England Biolabs, Beverly, MA). The radiolabeled and digested products were resolved by electrophoresis through a 2% agarose gel or an 8% or 10% polyacrylamide gel. The gel was dried, and the amount of radioactivity associated with each band was determined using a phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Positive controls for identification of specific mrp/plf cDNAs
were amplified from pRSV-plf1 or pRSV-plf2 cDNAs, or were produced using mRNA isolated from COS cells that had been transiently transfected with pCMV-MRP3. The diagnostic RT-PCR was validated using known mixes of \textit{mrp/plf} cDNAs followed by amplification and diagnostic RT-PCR. The amount of each cDNA was then determined quantitatively after restriction digestion and found to reflect the expected ratio of each cDNA.

Other \textit{mrp/plf} primers used for RT-PCR in these studies were: DAP1 (-230 with the addition of CCAC at the 5' end; 5'CCACTAGTCAGAGCATGAACAT3') and M3V55 (-129; with the addition of CCACTAG at the 5' end 5'CCACTAGTCATGGTATCTAGGCTACTTS').

Cloning and sequencing of the \textit{mrp4} cDNA The \textit{mrp4} cDNA was cloned from day 13 CF-1 mouse placenta. Total placental RNA was isolated and reverse transcribed as described under \textit{RNA isolation and RT-PCR}. The fragment was amplified by PCR using the following primers: DE1; 5'TAAGCCTGGGTAGGACTCTGC3' (+45 to +65), ZMRPUE5; 5'GAAAAGGTTGTCAACAACAAATTCAAAAG3' (+820 to +801). Twenty-seven cycles of 95 C (30 sec), 64 C (1 min) and 72 C (1.5 min) were performed using a low error rate amplification kit (Expand High Fidelity PCR System, Boehringer Mannheim, Indianapolis, IN). The PCR product was precipitated with ethanol, then digested 5' and 3' of the coding regions using PstI and HincII restriction endonucleases respectively. The resulting cDNA fragment was ligated into pBKSII+ that had
been cut with these same restriction endonucleases. The plasmid pBKS-mrp4 was sequenced by the DNA Synthesis and Sequencing Facility at Iowa State University to verify the sequence of the mrp4 cDNA insert. Two of the clones were sequenced. Because the two sequences differed in sequence at five base positions, total mRNA isolated from two different tail samples were amplified by RT-PCR and the amplicons sequenced. The resulting sequences matched each other and clone 2 with the exception of a single base at position 221 in clone 2 which was A. The base in this position in the two amplified total tail RNA samples was G, which matched that of the other three cloned mrp/plfs. The base at position 221 was therefore taken to be G because the amplified tail mRNA sequences were the sum of sequences of many mRNAs in each sample whereas the cDNA clone 2 was the result of a single copy of one mRNA.

Expression of MRP4 in COS cells The mrp4 cDNA was subcloned into pcDNA3 by digestion of pBKS-mrp4 with Smal and Xhol and insertion of the mrp4 cDNA into the blunt-ended HindIII and Xhol sites of pcDNA3 to make pcDNA3-mrp4. pcDNA3-Plfl was made by cloning the HindIII/Xbal fragment of pRSV-Plf 1 into the same sites in pcDNA3. COS cells were transiently transfected with pcDNA3-MRP4 or pcDNA3-PLF1 using DEAE-Dextran as co-precipitant (27). After transfection, the cells were grown in DMEG 10% calf serum for 48 h, then switched to 2% calf serum with or without 5 μg/ml tunicamycin for 24 h. The culture medium was collected and spun in a
microcentrifuge for 1 min and then stored at 4 C. The cells were scraped into lysis buffer (0.5% NP40, 100 U/ml Trasylol, 10⁻⁵ M PMSF, 20 mM HEPES, pH 7.25) and stored frozen at -70 C.

**Western blot analysis** Tissue homogenates for Western blot analysis were prepared as follows: halves of two or more placentae from pregnant mice were collected on either day 11 or day 14 of gestation, pooled according to day of gestation and homogenized in the same lysis buffer used for cultured cells above. Large tissue debris were spun out, and the supematants were saved. Transfected COS cell lysates or homogenates from day 11 or day 14 placentae were separated on a 15% polyacrylamide-SDS gel as described previously (28). The proteins were electroblotted to nitrocellulose membranes (Nitrobind; Micron Separations, Inc., Westborough, MA), and the MRP/PLFs were detected using rabbit anti-MRP/PLF antiserum at a dilution of 1/200, followed by treatment with horseradish peroxidase-conjugated protein A (Sigma) and Western blotting detection reagents (Amersham International, Buckingshire, England).

**Immunohistocemistry** Tissues were fixed in 4% paraformaldehyde in PBS (0.14 M NaCl, 2.7 mM KCl, 4 mM Na₂HPO₄, 14.7 mM KH₂PO₄, pH 7.4) for 1-2 h at 4°C immediately after collection. Samples were then rinsed in PBS and stored in 70% ethanol until sectioned. For immunodetection of MRP, 6 μm sections were rehydrated and stained as previously described (29) using a polyclonal rabbit anti-MRP/PLF serum (89rb13a) or a pre-immune serum from
the same animal, each at a dilution of 1/500. Primary antibody was detected using biotinylated goat anti-rabbit and horse radish peroxidase conjugated to avidin, and visualized by a peroxidase substrate, diamino benzidine tetrahydrochloride (ABC kit; Vectastain; Burlingame, CA). Samples were counterstained with hematoxylin and eosin, dehydrated, and mounted with permount (Fisher Scientific, Pittsburg PA) with a coverslip on top.

Identification of the promoters for the different mrp/plf genes Cosmid clones containing mrp/plf-hybridizing sequences and a reconstructed mrp3 gene from -1450 to +600 basepairs past the poly A site and genomic DNA were amplified using primers that recognize the sequence from +310 to +335 (exon 3; 5'ACAAAAGCCCATGAGATGCAATAC3') and from +455 to +435 (exon 4; 5'ACTCACTAGATCGTCCAGAGG3'). PCR-amplified fragments were digested with EcoRI for 4 h at 37 C and then digested with either BsoFI or BstXI as described for diagnostic RT-PCR. The fragments were resolved through a 2% agarose gel. Individual clones that produced diagnostic PCR digestion patterns consistent with a particular mrp/plf cDNA were sequenced across the promoter region using a primer +71 to +43 (UE1; 5'CATCTCTGAGTCAGCTACCCAGGCTTAG3') that hybridizes with the 5' end of mrp4 and the three mrp/plfs identified previous to this report (21-23).
RESULTS

A new mrp/plf mRNA that is expressed in the midgestational placenta

Because the placenta was previously the only tissue in which mrp/plf expression was observed, we used the diagnostic RT-PCR protocol diagramed in Fig. 1 to determine the relative proportions of mrp/plf mRNAs expressed in the placenta. By incorporating $^{32}$PdCTP into the final round of amplification we were able to quantitatively determine, using a phosphorimager, the amount of each restriction fragment. The major form of mrp/plf expressed during gestation was mrp3. This can be seen visually in Fig. 1 in which the majority of the radioactivity is associated with the 289 and 246 fragments that are diagnostic for mrp3. Less radioactivity was associated with the 318 bp fragment that is diagnostic for plf and no radioactivity was associated with the 275 bp fragment diagnostic for plf2.

However, we also found a form of mrp/plf mRNA that was not digested at the internal BsoF1 and BstX1 sites present in the three cloned mrp/plfs. The undigested form of mrp/plf was found in the placenta at lower levels before day 11 and peaks between days 12 and 14 (Fig. 1) (14). These results suggested the presence of a fourth, previously unidentified, mrp/plf mRNA that is expressed in the placenta during late gestation that does not contain the internal BsoF1 or BstX1 sites present in the three previously cloned mrp/plfs.
Cloning of \textit{mrp4}

The fourth \textit{mrp/plf} cDNA that was identified as uncut by the diagnostic RT-PCR procedure was cloned from day 13 placental RNA and sequenced as described in Materials and Methods to reveal a previously unknown \textit{mrp/plf} cDNA \textit{(mrp4)} with 91\% amino acid identity to \textit{plf1} (Fig. 2). Two noteworthy differences between \textit{mrp4} and the other three \textit{mrp/plfs} are changes in amino acids 77 (Ser to Pro) and 88 (Asp to His) that would disrupt two of the three putative N-glycosylation sites found on the other three MRP/PLF mature protein sequences.

Other possibly significant alterations include a stretch of five amino acids from 103 to 107 in which there are four differences between MRP4 and PLF1. Also, there is an in-frame deletion of two amino acids 167 and 168. Secondary structure analysis by NNPREDICT query (30) predicts that the substitutions in MRP4 at positions 186 and 188 result in the loss of helical tendency in the four alpha carbons between 185 and 188.

\textit{Mrp4} expression in the placenta

Among the nucleotide sequence differences between the \textit{mrp/plfs} is the presence of a Nde I site at +649 that is unique to \textit{mrp4}. To obtain further evidence that the \textit{mrp4} gene accounts for the uncut portion of cDNA observed
during later gestation, rather than a different *mrp/plf* that also lacks the Bsofl/Bstxl sites, diagnostic RT-PCR was carried out on placental RNAs using Ndel in place of Bsofl and Bstxl. Measured in this way, *mrp4* is found at higher relative levels on days 13-15 than days 9-11 of gestation as was shown previously with the Bsofl/Bstxl cleavage (Fig. 1) (14). Thus, both diagnostic RT-PCR methods identify the same gene product, *mrp4*, as having a different expression pattern relative to *plf1* and *mrp3*.

Measurements of the relative amounts of *plf1*, *mrp3* and *mrp4* in the placenta as a function of gestation showed that the proportion of *mrp3* varied between about 50% and 75% of the total *mrp/plf* mRNA throughout gestation. The relative amounts of *plf1* and *mrp4* varied reciprocally (Fig. 3). The maximum proportion of *mrp4* was reached on day 12 when it constituted about 40% of the *mrp/plf* mRNA in the placenta. At this time, the proportion of *plf1* is at its lowest of about 13% from a high of about 35% on day 7 of gestation.

**MRP4 is glycosylated differently from PLF1**

To determine how the loss of two potential N-glycosylation sites affects the glycosylation of MRP4, COS cells were transiently transfected with pcDNA3-MRP4 or pcDNA3-PLF1. Culture media and lysates of the transfected cells were analyzed by Western blot and compared with the MRP/PLFs found in placental homogenates (Fig. 4). As expected, PLF1 migrated with an apparent molecular
weight ranging between 32K and 36K (13). By comparison, placental minces secrete MRP/PLFs of 27 kDa and 38 kDa. The 38-kDa MRP/PLF band was previously identified as containing MRP3 and PLF1 (14). MRP4 migrated at the same rate as the 27-kDa placental MRP/PLF. The 27-kDa MRP/PLF has a time course of appearance in gestation similar to that of the mrp4 mRNA.

PLF1 and MRP4 are polypeptides having the same apparent molecular weight. This is demonstrated by a comparison of the nonglycosylated forms of the two proteins (Fig. 4). The nonglycosylated forms of the MRP/PLFs, produced by transfected cells that were incubated with tunicamycin, migrated as expected for the 21K molecular weight proteins predicted from their cDNA sequences. These results demonstrate that the difference in electrophoretic migration rates between the two MRP/PLFs is due to their glycosylation. Thus, the loss of two glycosylation sites in the MRP4 sequence results in the addition of less carbohydrate to this protein compared with PLF1. The placental MRP with the same apparent size as the recombinant MRP4 produced by COS cells is more predominant in day 14 placental lysates than day 11 lysates. This is consistent with the delayed expression of MRP4 mRNA relative to PLF1 and MRP3 in the placenta (Fig. 3) (14).
Differential cell and tissue-specific expression of *mrp/plf* genes.

Although *mrp/plfs* were initially demonstrated by Northern blot analysis to be expressed only in the placenta (6), we show here that the *mrp/plfs* are differentially expressed in a variety of mouse tissues (Fig. 5). *Mrp/plf* mRNAs were detected in the small intestine and tail but not in the liver, lungs, backskin, adult ribs or large intestine. The same expression pattern was found in males and females. *Mrp/plfs* were detected in some, but not all, stomach samples and at low levels in some samples of backskin (data not shown). *Mrp/plfs* were also detected in 3T3 cells as expected from previous studies (13). The major *mrp/plf* expressed by 3T3 cells is *plf1* consistent with the cloning of this *mrp/plf* from these cells (21). Samples incubated without reverse transcriptase showed no visible bands (Fig. 5A). Restriction analysis of radiolabeled cDNAs showed that the mouse tail produces only *mrp4*, while the small intestine and 3T3 cells produce mostly *plf1* with lower levels of *mrp3* (Fig. 5B). *Mrp4* seems to be expressed at lower relative levels in the small intestine, but is absent from 3T3 cells. The PCR product from cDNA reverse transcribed from whole tail RNA was sequenced, and the results confirmed that the *mrp/plf* sequence of the mRNA expressed in the tail is the same as that of the *mrp4* cDNA cloned from day 13.5 placentae.
The mouse tail skin and the mouse ear skin express unique keratins that are different from those found in normal backskin. Therefore, RNA from the ear also was subjected to diagnostic RT-PCR and compared with the results from tailskin RNA (Fig. 5B). Although mrp/plf cDNA was not detected in most normal backskin samples (Fig. 5A), mrp/plf cDNAs were regularly identified in both the ears and tailskin where mrp4 was the only identified mrp/plf (Fig. 5B). Mrp4 also was the major mrp/plf species detected in the remainder of the tail after the skin was removed (data not shown).

Identification of the mrp4 promoter

To identify the mrp4 promoter, a series of cosmid clones containing mrp/plf sequences were screened for the presence of the mrp4 gene by several PCR-based assays. First, diagnostic PCR was performed between exons 3 and 4 on isolated cosmid clones containing mrp/plf hybridizing genes in order to identify specific mrp/plf-containing cosmids. Individual clones were identified that produced the predicted restriction digestion pattern for each of the mrp/plfs. Clear restriction patterns were obtained for five cosmid clones; two cosmids gave patterns specific for plf1 (cos4 and cos5), two gave mrp3-specific patterns (cos2 and cos3) and one gave the mrp4 restriction pattern (cos6). Clones that produced PCR products with mrp/plf-specific digestion patterns were also sequenced across the promoter region after PCR amplification starting with a
primer in the common 5'UTR. By this method, the promoter known as plf42 was shown to be contiguous with the gene encoding plf1, the promoter plf149 corresponded to mrp4 and the mrp3 promoter corresponded to mrp3 cDNA.

To confirm the promoter cDNA contiguities deduced from the cosmid clones, RT-PCR was performed using 5' primers that would amplify transcripts initiating at upstream promoters located between 140 or 230 basepairs of the normal transcriptional start site (data not shown). When performed on RNA from the tail (using primers DAP1 and NUE5), the promoter for mrp4 was confirmed to be plf149. Similarly for 3T3 cells, which express predominantly plf1, RT-PCR using M3V55 and UE5 produced upstream transcripts that corresponded to the plf42 promoter. As expected, the placental mrp3 cDNA was found to be linked to the mrp3 promoter when RT-PCR was performed using DAP1 and NUE5.

**Localization of MRP4 in the hair follicles of the mouse tail**

To determine the location of the MRP4 protein in the adult tail, immunohistochemistry was performed using a rabbit polyclonal anti-MRP/PLF serum. Binding of anti-MRP/PLF antibody was detected in the hair follicles of the tail skin (Fig. 7). The location of MRP/PLF in the hair follicles appeared to be limited to the keratinocytes that make up the outer root sheath. Pre-immune sera did not stain these structures.
DISCUSSION

The MRP/PLFs exhibit several very important biological activities. They stimulate proliferation, angiogenesis and inhibit differentiation. However, until this report, they have only been demonstrated to be expressed in the placenta. There are between four and six mrp/plf genes in the mouse (20) and three of the cDNAs have been previously identified. Here, we describe the identification and cloning of a fourth mrp/plf (mrp4) that is transiently expressed during gestation in a manner distinct from the other mrp/plfs and which is uniquely expressed in the tail and ear of adult mice.

MRP4 is more different in sequence from the other three MRP/PLFs than are the other three MRP/PLFs from each other. Whereas MRP4 is 91% identical with PLF1, the other three MRP/PLFs (PLF1, PLF2 and MRP3) are 99% identical with each other. The MRP4 protein also is differently glycosylated because two differences in the amino acid sequence result in the loss of two potential N-glycosylation sites from MRP4 compared with the other MRP/PLFs. This difference in glycosylation might alter the stability of the protein, its interaction with receptors or its ability to move from one tissue to another. For example, we have demonstrated that the 27-kDa form of MRP/PLF in the placenta that we identified as MRP4 is not found in the bloodstream or the amniotic fluid of the pregnant mouse as are other MRP/PLFs (14).
Mrp4 expression peaks on day 12 of gestation. This is at a time during which expression of the other two mrp/plfs is decreasing (6, 14, 31). Interestingly, it is the same time at which proliferin-related protein (PRP) peaks (32). PRP is closely related in sequence to the mrp/plfs with 37% identity to PLF1 in amino acid sequence and 95% identity to PLF1 in the first 97 nucleotides (6). PRP opposes the effect of bFGF on angiogenesis, and the delayed increase in PRP expression in the placenta is proposed to effect a rapid inhibition of the angiogenic effects of the MRP/PLFs (16). Similar to MRP4, PRP is not found in the amniotic fluid, but unlike MRP4, PRP is found in maternal serum (32).

Individual members of the mrp/plf family are differentially regulated in vivo in both tissue-specific and temporal patterns. As well as being expressed in the placenta, the mrp/plfs are expressed in the small intestine, tail, and ear and sometimes were observed in the stomach. Although not detected in most samples of backskin, occasionally samples showed low levels of mrp/plf expression. This expression is mainly of mrp3 (Fassett and Nilsen-Hamilton, manuscript in preparation). The mrp/plfs were not detected in the liver, lungs, ribs, and large intestines. Different mrp/plf genes are expressed in different tissues. Plf1 is the major form of mrp/plf mRNA expressed in the stomach, small intestine and 3T3 cells, while mrp3 is the major form expressed by the midgestational placenta. Mrp4 is the only form of mrp/plf expressed in the ear
and tail skin. As we have shown a broader tissue expression pattern of the mrp/plfs beyond the placenta which is their site of highest expression, so are other genes of the prolactin/growth hormone family found to be expressed in high levels in one primary tissue and secondarily in other tissues. Beyond its primary site of expression in the pituitary, prolactin, the evolutionary precursor to the rodent utero/placental gene family, also is expressed in other tissues, including the dermis (33). The placental lactogens I and II are expressed in the testes in addition to their high levels of expression in the placenta (34).

By immunohistochemistry, the MRP4 protein is localized in the keratinocytes of the outer root sheath of the hair follicle in the tail. These cells may not be the site of the protein's initial synthesis, but they are apparently the site of highest accumulation of MRP4. Thus, it is likely that the keratinocytes are either the source of MRP4 or the site of its action or perhaps both.

The observation that the mrp/plfs are expressed in a tissue-specific manner suggests that these genes are regulated by distinct factors. As well, the site of expression of MRP4 is specific to the location in the body, e.g., tail vs. back. This observation parallels those of the keratin gene family where the keratin pair of 48 kDa and 65 kDa is expressed specifically in suprabasal cells of the tail epidermis, in the filiform papillae of the tongue, and at low levels in suprabulbar cells of newborn hair follicles (35). In mice, a 70-kDa keratin (mk2e) is
expressed specifically in the ear, tail and footsole epidermis (36). The ear and tail are also the specific sites of \textit{mrp4} gene expression.

Here we have shown that the promoter for \textit{mrp4} is the previously cloned plf149 promoter and the plf42 promoter as being the sequence upstream of the \textit{plf1} coding sequence (37). Although 98% homologous to the promoter for \textit{plf1} (plf42), the \textit{mrp4} (plf149) promoter is not stimulated by the addition of serum (37) or bFGF (38). Interestingly, there are a number of differences between the plf149 and \textit{mrp3} and plf42 promoters that affect recognizable regulatory elements. One of these changes affects the FGF response element (FRE) present in \textit{mrp3} which is disrupted in the other two promoters (38). This and other alterations in regulatory sequences are likely to be responsible for the differential expression of the \textit{mrp/plfs} in mouse tissues.

In summary, we have identified a fourth \textit{mrp/plf} gene with a distinct tissue expression pattern from the other \textit{mrp/plf} genes. Despite the high identity in nucleotide sequence, it is striking that a large number of the differences in nucleotide sequence of the first 550 bp of these promoters result in alterations of recognizable regulatory element sequences. These observations suggest that the \textit{mrp/plf} family may have evolved to take advantage of specific combinations of transcriptional regulatory elements for the regulation of \textit{mrp/plf} gene expression in particular cell types or under special physiological conditions. The resulting MRP/PLF proteins, which are also 91% to 98% identical in amino acid
sequence, may not differ in their function. However, there may be other differences. For example, the difference in glycosylation of MRP4 compared with MRP3 and PLF1 could result in altered stability or tissue access for MRP4, or MRP4 may interact with a different set of receptors. Thus we suggest that MRP4 may function as a growth factor and/or angiogenesis factor in the vicinity of the hair follicle in the tail and ear as MRP3 and PLF1 are proposed to do in the placenta.

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FIGURES

Figure 1. Diagnostic RT-PCR of mrp/plf cDNAs. Top panel. Diagram to show how digestion with the restriction enzymes BsoFI and BstXI results in different sized restriction fragments for each mrp/plf. Resolution of these fragment by agarose or acrylamide gel electrophoresis followed by their quantitation provides estimates of the relative amounts of each mrp/plf mRNA in individual samples. Bottom panel. The mrp/plfs are distinguishable by their digestion pattern. Plf1, plf2, and mrp3 cDNAs and placental cDNA from various gestational days were amplified and digested with BsoFI and BstXI as described in panel A. The resulting fragments were run on an 8% polyacrylamide gel. The top arrow indicates the 564-bp fragment that does not digest at either internal restriction site.

Figure 2. Sequence comparisons of MRP4 with other MRP/PLFs. The nucleotide sequence of plf1 is compared with that of mrp4. The complete protein sequence of MRP4 is shown and sequences of the other three proteins are shown only where they differ from MRP4. The signal sequence is underlined. The amino acids that are changed in the N-glycosylation sites are italicized. The three putative glycosylation sites are identified by the boxes. The numbers to the left of the protein sequences show the position of the first amino acid in each row in the PLF1 protein sequence. Note that after position 166, the MRP4 sequence
is two amino acids short of the other three sequences due to the deletion of amino acids 167 and 168 from the MRP4 sequence.

Figure 3. Placental expression of the mrp/plf s. A, upper panel:
Quantitation by diagnostic RT-PCR of the proportional expression of plf1, mrp3, and mrp4 by the placenta through gestation. These data were also used, along with the total amount of RNA determined by Northern blot analysis, to determine the relative amounts of each of the mrp/plf genes in gestation (13). The results shown are the average of four separate experiments in which different placental samples were analyzed. The results were averaged to obtain the results shown. As a result of different combinations of samples in each experiment, the values are the average of two (days 8,10,12,13) or three (days 9,11,15) separate values. Shown are the sample standard deviations. A, lower panel: The expression of mrp4 was analyzed during gestation using the restriction endonuclease Nde1, which digests mrp4, but not the other identified mrp/plf s. Shown are averages of two experiments. B. Digestion pattern of the mrp/plf  amplicons with Nde1.

Figure 4. Western blot of MRP/PLFs produced by transiently transfected COS cells compared with the MRP/PLF proteins present in the placenta. COS cells were transiently transfected with mammalian expression plasmids for MRP4 (4) and PLF1 (1) as described in Materials and Methods. Some cells (1T, 4T) were treated with 5 μg/ml tunicamycin for the last 24 h of incubation.
Medium from the transfected cells and cell lysates were resolved by SDS-PAGE.

1, COS cells transfected with pcDNA3-PLF1; 4, COS cells transfected with pcDNA3-MRP4; 1T, 4T, tunicamycin treated cell cultures transfected with pcDNA3-PLF1 or pcDNA3-MRP4 respectively; e11 and e14, homogenates of day 11 and 14 placentae respectively. The positions of three molecular weight markers, ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and myoglobin (18 kDa) are shown. The positions of PLF1 and MRP4 in cells and culture medium are marked on the left of the figure and the positions of PLF1 and MRP3, and MRP4 in the placental homogenates are noted on the right of the figure. The position of the nonglycosylated forms of PLF1 and MRP4 is also shown (NG).

Figure 5. Diagnostic RT-PCR demonstrates mrp/plf expression in several murine adult tissues. A. (top three panels) Mrp/plf sequences in RNA isolated from 2-month old CF-1 mouse liver, lungs, skin, tail, ribs, small intestine, large intestine, and 3T3 cells were amplified by RT-PCR as described in Material and Methods. A control of water alone was also included. The initial reaction to create mrp/plf cDNA was done in the presence (+RT) or absence (-RT) of reverse transcriptase. The third panel down shows the result of amplifying with primers for G3PDH. B. (bottom panel) Mrp/plf cDNAs from e11 placenta, adult tail, ear, adult small intestine, and 3T3 cells were digested with BsoFI and BstXI. Shown are the results from two experiments with the two lanes on the right from the second experiment. The tail samples in the two experiments were from
different animals. The results from digestions of the fragments isolated from
plf1, plf2, mrp3 and mrp4 standard samples are also shown.

Figure 6. Immunodetection of MRP in the tail of the adult mouse. Sections of tail
from a non-pregnant female FVB mouse were stained with anti-MRP/PLF antibody
(A, top panel) or pre-immune sera (B, bottom panel). Antibody stains the outer root
sheath of the hair follicles (brown color, top panel). Similar results were obtained for
male and female, CF-1 and FVB mice.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
CHAPTER 4: MRP3, A MITOGEN-REGULATED PROTEIN/PROLIFERIN GENE EXPRESSED IN WOUND HEALING AND HAIR FOLLICLES

A paper to be submitted to the journal Endocrinology

John T. Fassett and Marit Nilsen-Hamilton

ABSTRACT

During cutaneous wound healing, a marked increase in the local expression of growth factors results in increased migration and proliferation of the cells responsible for tissue repair. The MRP/PLF Mitogen regulated protein/proliferin) proteins are growth factors and angiogenesis factors. Here it is demonstrated that \textit{mrp3} is induced in wound edge keratinocytes during cutaneous wound healing and also temporally appears in the outer root sheath of the hair follicle during the late anagen phase of the hair cycle. In cultured keratinocytes, \textit{mrp3} is induced by keratinocyte growth factor but not by epidermal growth factor or transforming growth factor type \(\alpha\). Transgenic mice, expressing lacZ under the combined control of the cytomegalovirus immediate early (CMV-IE) enhancer

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and the \textit{mrp3} flanking sequences, demonstrate wound and hair cycle-induced transgene expression, showing that elements within the flanking regulatory sequences of the \textit{mrp3} gene are involved in the activation of \textit{mrp3} in response to these events. The results reported here suggest that \textit{mrp3} may participate in wound healing and in the hair follicle cycle as a growth factor and/or angiogenesis factor.

\textbf{INTRODUCTION}

A hallmark of wound healing is the release of growth factors at levels much higher than are typically found in nonwounded skin (Wemer \textit{et al.}, 1992). Fibroblast growth factor (FGF) family members stimulate angiogenesis (Clark, 1996), as well as migration and proliferation of fibroblasts and keratinocytes (Tsuboi \textit{et al.}, 1992; Werner, 1998). Expression of a dominant negative FGF7 receptor in the skin of mice delays wound healing (Wemer \textit{et al.}, 1993). The application of bFGF enhances wound healing in healing-impaired db/db mice (Tsuboi and Rifkin, 1990). Thus, re-epithelialization and tissue repair are likely to be mediated in part by the ability of skin cells to appropriately respond to FGFs and other growth factor signals.

Growth factors, including FGF5, FGF7, EGF, and TGFα are also involved in hair follicle morphogenesis, as evidenced by the hair-associated abnormalities

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linked to mutations or introduced knockouts of these genes or their receptors (Guo et al., 1996; Hebert et al., 1994). The hair follicle contains stem cells that are likely to contribute to cutaneous wound healing by providing a source of the keratinocytes that migrate and proliferate to repair the wound (Cotsarelis et al., 1990).

The mitogen regulated protein/proliferins (mrp/plfs) are a group of highly homologous, well characterized growth factor-inducible secondary response genes and members of the prolactin/growth hormone family (PRL/GH). In immortalized mesenchymal cells derived from mouse embryos (3T3 cells), mrp/plf expression is increased in response to bFGF and other growth factors (Nilsen-Hamilton et al., 1980). Expression of mrp/plf is greater in growing than in quiescent cells (Linzer and Wilder, 1987). This may be in part explained by the fact that transforming growth factor type β (TGFβ) inhibits mrp/plf expression in quiescent but not in growing cells (Chiang and Nilsen-Hamilton, 1986).

There are between 4 and 6 mrp/plf genes (Jackson-Grusby et al., 1988), and the four cloned cDNAs (plf1, plf2, mrp3, and mrp4) are highly identical to one another (Connor et al., 1989; Fassett et al., 2000). Although some non-placental cell lines express mrp/plfs in vitro, the only tissue identified in the mouse in which mrp/plf expression was identified until recently is the placenta (Linzer et al., 1985). Mrp/plf expression is localized to the trophoblastic giant cells, which secrete high levels of mrp/plf during mid-gestation (Lee et al., 1988;
Nilsen-Hamilton et al., 1988). Placental mrp/plf is believed to be an angiogenesis factor in the fetal placenta and activates the mannose 6-phosphate receptor (Jackson et al., 1994). It is also a growth factor for the uterus that acts through a separate receptor in the uterus that does not recognize mannose 6-phosphate (Fang et al., 1999; Nelson et al., 1995). Thus, the MRP/PLFs are believed to coordinate aspects of fetal and maternal development during pregnancy through their ability to regulate cell proliferation and angiogenesis.

Because growth factors are expressed at high levels during wound healing, and the mrp/plfs are highly upregulated by growth factors in 3T3 cells, we investigated the possibility that mrp/plf expression might also be increased during wound repair. Here we demonstrate that mrp3 is expressed specifically in suprabasal keratinocytes at the wound site, and its expression is regulated temporally during cutaneous wound healing. MRP/PLF protein and RNA expression are also regulated during the hair follicle cycle, where immunohistochemistry reveals the protein predominantly in the outer root sheath. The same distribution and regulation of expression was found in transgenic mice expressing a lacZ transgene under combined control of the cytomegalovirus immediate early (CMV-IE) enhancer and the mrp3 flanking sequences. We also show that mrp3 expression is induced by KGF in primary cultures of keratinocytes. These data suggest that mrp3 plays a role during cutaneous
wound repair and in the hair follicle cycle. A likely regulator of mrp3 gene expression in the skin is KGF.

RESULTS

Mrp/plf Genes Are Expressed in Regenerating Epidermis

To determine if the MRP/PLFs are produced during wound healing, full thickness wounds were created on the backs of mice and, at various times after wounding, immunohistochemistry was performed on the wounded skin tissue by using anti-MRP/PLF. Antibody staining was most evident in the suprabasal keratinocytes at the wound edge between days 4 and 5 after wounding (Fig. 1A). Staining was less evident before hyperproliferation of wound edge keratinocytes, which occurs on about day 4, and was also less intense as the wound became reepithelialized after day 7.5 (data not shown). There was little staining in basal keratinocytes. Stain was occasionally found over the outermost terminally differentiated keratinocytes. Often, keratinocytes expressing MRP/PLFs were located along the hair follicles near the wound. Staining was also occasionally detected in the outer root sheath of hair follicles not associated with the wound (not shown). There was no stain using pre-immune sera (Fig. 1B).
Mrp3 Is the Major Mrp/plf Gene Expressed During Wound Healing

To determine whether mrp/plf genes are expressed in wounded tissue, total RNA was extracted from wounds at different times after wound healing and subjected to RT-PCR. G3PDH was also amplified from the same cDNAs in parallel analyses to serve as controls for sample quality. Mrp/plf mRNA was detected in wound tissue after one day of wounding and seemed to be present at the highest levels at 2.5 days after wounding (Fig. 2A).

To determine which mrp/plfs are expressed during wound healing, the four known mrp/plf gene products were distinguished by diagnostic RT-PCR as described in Materials and Methods. A portion of each sample shown in Fig. 2A was amplified for an additional single round in the presence of $^{32}$P-dCTP, digested with Bsof1 and Bstx1, and separated on a nondenaturing 10% polyacrylamide gel. Standards for individual mrp/plf forms were treated similarly and used as controls for the expected fragments. As shown in figure 2B for day 2.5, mrp3 makes up most of the mrp/plf mRNA found in skin wounds.

Keratinocyte Regulation of Mrp/plf Expression in Response to Growth Factors.

To better understand which growth factors may be inducing mrp/plf expression at the wound edge, newborn mouse keratinocytes were isolated and
treated with KGF (FGF7), EGF, or TGFα. Mrp/plf gene expression, as measured by Northern blot analysis, was increased in response to KGF, whereas EGF and TGFα had no effect (Fig. 3A, B). As in the wound, mrp3 was the major form of mrp/plf expressed by FGF-stimulated mouse keratinocytes although some plf1 was also expressed (Fig. 3C). By comparison, 3T3 cells expressed mostly plf1.

**Mrp3 Transgene Expression in Response to Wound Healing**

Transgenic mice carrying the CMV/MPR3/lacZ/3U DNA construct were examined for β-galactosidase expression in response to wound healing. In full thickness cutaneous wounds in the back, staining was evident between 1 and 3 days after injury in one of two independently isolated transgenic lines containing the complete CMV/mrp3 construct (Fig. 4A) and in two independent transgenic mouse lines containing a CMV/MPR3/lacZ construct that contained about 600 bp instead of ~3kb of 3' mrp3 UTR. One of the latter two lines also showed staining in the panniculous carnosis. Both the location and the time course of transgene expression was similar to endogenous mrp3 expression.

In all four transgenic lines containing the CMV/MPR3/lacZ construct with ~600bp or ~3' 3kb of mrp3 sequence, tail injury resulted in expression of lacZ specifically in the skin around the edge of the wound (Fig. 4B). No staining was observed in tissue fixed immediately after wounding (Fig. 4C). In a control transgenic mouse line containing the CMV-IE enhancer but only 49bp of the
mrp3 promoter, lacZ was expressed constitutively throughout the skin and in most other organs tested as well (not shown).

**Presence of MRP/PLFs in the Hair Follicle**

In the course of these studies it was noticed that some hair follicles also stained for MRP/PLF. The protein was detected by immunocytochemistry using anti-MRP/PLF but not with preimmune serum (Fig. 5A,B). MRP/PLF staining was strongest in the outer root sheath of the hair follicle and absent from the bulb. The morphology of the follicles that were stained with anti-MRP/PLF indicated they were in the anagen stage of the hair follicle cycle.

**Regulation of Mrp/plf Genes During Hair Follicle Morphogenesis**

To analyze expression of the mrp/plf genes during the hair follicle cycle, black coated C57/BL6 x CF-1 hybrid mice were depilated and skin samples were collected at various times afterwards. Staining was evident in some follicles in the outer root sheath during mid-anagen (day 8) and was more extensive at late anagen (day 17.5) of the hair follicle cycle. No staining was evident when preimmune serum was used (Fig 5C-H). Staining was also seen in the outermost epidermal layers.

RNA isolated from other tissue samples in the same experiments were analyzed by diagnostic RT-PCR (Fig. 6). The expression of mrp/plfs was highest by day 17.5 (Fig. 6A) which corresponds to late anagen (Pena *et al.*, 1999).
Mrp3 was the predominantly expressed mrp/plf during normal hair follicle cycling (Fig. 6B). Smaller proportions of plf1 and mrp4 were also expressed.

The CMV/MRP3/lacZ/3U transgenic mice showed strong β-galactosidase staining in the outer root sheath of anagen hair follicles (Fig. 7A). Staining in the more external elements of the hair follicles was generally darker and more consistent than staining near the bulb region. This could have been due to limitations in the penetration of the X-gal through the tissues. Consequently, the distribution of β-galactosidase expression was also determined in hair follicles that had been plucked from the animal prior to staining (Fig 7B).

DISCUSSION

For many years, the only reported in vivo sites of mrp/plf expression were the trophoblastic giant cells of the placenta (Lee et al., 1988). Recently we have demonstrated the selective expression of one member of the mrp/plf family known as mrp4 in the hair follicles of the tail (Fassett et al., 2000). Here we show that another member of this family, mrp3 is selectively expressed in keratinocytes during wound healing and in the late anagen hair follicle.

Mrp3 gene expression starts soon after wounding although the MRP3 protein is not detected by immunohistochemistry until it has accumulated to high levels 4-5 days after wounding. The increase in mrp3 gene expression revealed by RT-PCR begins early in the inflammatory stage and continues through the
granulation stage of wound healing, the latter being the period of keratinocyte proliferation and the re-epithelialization phase of wound healing (Clark, 1996).

Keratinocyte growth factor (KGF, also FGF-7), a potent keratinocyte mitogen (Werner, 1998) increases \(mrp3\) expression in keratinocytes in culture. In vivo, dominant negative KGF receptors cause severe defects in wound healing (Werner et al., 1994), whereas KGF ligand knockouts show normal wound healing but have abnormal coats (Guo et al., 1996). These results suggest that signaling through KGF receptors is crucial for wound healing but the KGF ligand itself is not essential. This result is consistent with the observation that several members of the FGF family interact with \(fgfr2\), the KGF receptor.

Neither EGF nor TGF\(\alpha\) increased \(mrp/\text{plf}\) expression by keratinocytes, even though these are mitogens for keratinocytes and stimulate \(mrp/\text{plf}\) expression in 3T3 cells (Nilsen-Hamilton et al., 1980). Here it is relevant that \(\text{plf1}\) is the major \(mrp/\text{plf}\) gene expressed in 3T3 cells, and the predominantly expressed gene in keratinocytes is \(mrp3\). The \(mrp3\) promoter but not the \(\text{plf1}\) promoter is stimulated by FGF in 3T3 cells (Mohideen et al., 1999).

MRP3 was not detected in the keratinocytes found at the very surface of the wound that are believed to be migrating across the wound. In humans, the migrating keratinocytes display the highest level of immunostaining for the cleaved and activated form of TGF\(\beta\) (Kane et al., 1991). MRP/PLF expression by 3T3 cells is inhibited by TGF\(\beta\) (Chiang and Nilsen-Hamilton, 1986). Results
here showing that 3T3 cells express mostly plf1 suggest that these previous studies demonstrated that TGFβ inhibits the expression of plf1. However, in 3T3 cells stably transfected with 2 kb of the mrp3 promoter upstream from a CAT reporter gene, TGFβ also inhibited the mrp3 promoter activity (Mohideen and Nilsen-Hamilton, unpublished). Thus, mrp3 expression may be suppressed by TGFβ in migrating keratinocytes.

Although cultured keratinocytes are not physiologically equivalent to wounded skin, the regulation of mrp/plf in response to growth factors may be similar to that in vivo. KGF is highly expressed by the dermis from 24 h after wound healing through at least seven days (Werner et al., 1992). Mrp3 is expressed over this same time in the suprabasal keratinocytes at the wound margins. During wound healing, proliferation of the underlying basal layer results in a greatly thickened layer of suprabasal keratinocytes. Although mitotic figures are evident in the suprabasal layer, the majority of cell proliferation in the wound is in basal keratinocytes. MRP3 might be part of a positive feedback mechanism by which the suprabasal cells stimulate further proliferation of the cells in the underlying basal layer. This mechanism would parallel that of PLF1, a growth factor for primary uterine cells (Nelson et al., 1995).

The MRP/PLFs signal through two different receptor types: a unique MRP/PLF receptor (Nelson et al., 1995) and the IGF-II/mannose-6-phosphate receptor (Rogers et al., 1990; Volpert et al., 1996). In sheep, IGF-II receptors
are expressed in the germinal matrix with peak expression during late anagen/early catagen and in the dermal papilla with peak expression during telogen (Nixon et al., 1997). If the IGFII receptor is similarly expressed in the mouse, then the cells expressing these receptors are possible targets for the MRP/PLFs that are expressed in late anagen.

The FGF family stimulates proliferation and angiogenesis and are involved in cutaneous wound healing. Expression of a dominant negative FGF7 receptor in the skin of mice delays wound healing (Werner et al., 1993), and the application of bFGF enhances wound healing in healing-impaired db/db mice (Tsuboi and Rifkin, 1990). Some FGFs are also produced in hair follicles: Fgf5 and Fgf7 mRNAs are present in the outer root sheath during anagen IV (Hebert et al., 1994; Rosenquist and Martin, 1996). Messenger RNAs encoding several FGF receptors are also present in the hair follicle during anagen; Fgfr1 mRNA in the dermal papilla, Fgfr2 RNA in hair matrix cells near the dermal papilla, Fgfr3 mRNA in pre-cuticle cells in the periphery of the hair bulb, and Fgfr4 RNA in cells in the periphery of the hair bulb and the inner and outer root sheath in the lower half of the follicle neck. These FGFs and their receptors are likely to be active after the period of follicle growth because proliferative cells are found in the outer root sheath during catagen and telogen (Tezuka et al., 1990). KGF is specific for fgfr2 (Miki et al., 1992) but fgfr4 is expressed by the outer root sheath cells.
Thus, it is possible that an FGF other than KGF stimulates mrp/plf expression in these cells.

Expression of mrp/plf in the skin was confined to the wound keratinocytes and epithelial cells of the outer root sheath cells of the hair follicle. In these cells, mrp3 was the major form expressed. Mrp3 expression was also observed in skin containing hair follicles in late anagen where plf1 and mrp4 were also found in smaller relative amounts. The expression of small proportions of plf1 and mrp4 in hair follicles and not in healing skin could reflect the different mouse strains used in the wound healing (FVB) and hair cycle (C57/BL6 x CF-1 crosses) studies. However, we did not observe differences in mrp/plf types expressed in placental tissues from CF-1 and Balb/c mice (Fassett et al., 2000). Recently, we have also observed expression of predominantly plf1 in stomach epithelia (unpublished) and plf1 and mrp3 in the small intestine (Fassett et al., 2000), whereas mrp4 is constitutively expressed in the skin of the adult tail (Fassett et al., 2000). Thus, mice seem to have evolved mechanisms to express different mrp/plf forms in different epithelial tissues.

Transgenic mice containing a CMV/MRP3/LacZ transgene expressed β-galactosidase in the wound and hair follicles in the same cellular locations and with the same time course as seen by RT-PCR for endogenous mRNA expression and by immunocytochemistry for MRP/PLF protein localization. In these studies, the presence of the CMV-IE enhancer seemed to increase
expression from the mrp3 promoter. In other studies, the CMV-IE enhancer did not efficiently increase transgene expression in the skin from the CMV promoter (Sawicki et al., 1998). However, the CMV-IE enhancer in combination with the chicken β-actin promoter drove β-galactosidase transgene expression strongly in many locations in the skin with the highest expression observed in the sebaceous gland, epidermis, suprabasal and basal cells of the epidermis, and the arrector pili muscle. Expression was not high in the outer root sheath. Thus, the promoter (CMV or β-actin) rather than the CMV-IE enhancer determines the pattern of expression of the transgene (Sawicki et al., 1998). We show here a different expression pattern of the mrp3 promoter in combination with the CMV-IE enhancer than previously reported for CMV-IE enhancer with the CMV promoter or with the β-actin promoter. Although the presence of the CMV-IE enhancer increases the level of transgene expression from the mrp3 promoter, the information necessary to determine spatial expression of mrp3 in the wound and the hair follicle seems to be present in the 1,514 bp proximal mrp3 promoter. This transgenic construct also expressed specifically in the giant cells of the placenta during gestation which is the major site of mrp3 expression during development (Fassett and Nilsen-Hamilton, in preparation).

If the functions of mrp/plfs in the skin are similar to those proposed for mrp/plfs during reproduction then MRP3 might promote proliferation by way of
the MRP/PLF receptor (Nelson et al., 1995) and angiogenesis through the IGFII receptor/mannose-6-phosphate (Jackson et al., 1994).

The timing of mrp3 expression during the late anagen stage of the hair follicle cycle is not consistent with an immediate effect of this growth factor on angiogenesis because the perifollicular capillary bed regresses after anagen (Lachgar et al., 1996). However, although most cell division ceases in the hair follicle after anagen, there still appears to be proliferation in the outer root sheath (ORS) during telogen (Tezuka et al., 1990). Thus, the immediate role of MRP3 might be to stimulate ORS cell proliferation at a time when most cell proliferation in the hair follicle has ceased. However, a highly glycosylated and very stable protein such as MRP3, produced and secreted late in the hair follicle cycle, might be adsorbed to the proteins in the extracellular matrix and then released at some later time, perhaps directly prior to the anagen phase of the subsequent hair cycle when it could act as an angiogenic factor as well as a growth factor for the newly forming hair follicle.

In conclusion, we have shown that mrp3 is expressed in keratinocytes during wound healing and in the outer root sheath of the late anagen hair follicle. Members of the FGF family are the most likely positive regulators of mrp3 gene expression in the skin whereas TGFβ may be a negative regulator. Studies with transgenic mice suggest that the DNA elements necessary to direct mrp3 expression in the wound and in the hair follicle are present in the DNA.
sequences flanking the mrp3 coding sequence. On the basis of previous findings, we suggest that MRP3, previously identified only in the placenta, is a growth factor and an angiogenesis factor that may play a role during wound healing and the hair follicle cycle.

MATERIALS AND METHODS

**Animals and materials** CF-1 and FVB mice, originally obtained from Charles River (Wilmington, MA), were bred and cared for at the Laboratory Animal Facility at Iowa State University under a 12-h light/dark cycle. All animals were housed and treated according to current National Institutes of Health guidelines. Care was provided by an animal caretaker and an attending veterinarian. With the exception of the removal of tail segments, animals were killed by CO₂ asphyxiation before removal of tissues for the described studies. This research was conducted in accordance with the standards set forth in the NIH guide for the care and use of laboratory animals. Prior approval was obtained from the Iowa State University Committee on Animal Care for all procedures performed on the animals used in these studies.

Polyclonal anti-MRP rabbit sera and preimmune sera were prepared as described (Nilsen-Hamilton et al., 1987). Plasmid vector pckt17-2, a modified pSP73 (Promega, Madison WI) was a gift from Christopher Tuggle (Iowa State University). KGF was purchased from R&D Systems; Minneapolis, Minn).
Wound healing experiments: FVB mice (Charles River; Wilmington, MA) were used for most wound healing experiments except for those performed on transgenic animals, which were FVB or FVB/CF-1 crosses. To produce wounds, adult mice were anesthetized by using Avertin as previously described (Papaioannou, 1990), and scissors were used to make 0.5 to 1 cm full thickness skin wounds laterally along the back. Two equally spaced nylon sutures (5-0 ethilon; Ethicon; Somerville, New Jersey) were used to close each 1-cm wound in the back. In some cases the tip of the tail was cut off to produce a wound and for use in identifying transgenic offspring. At various times after wounding, the wound tissue was removed and either fixed for immunohistochemistry or frozen in liquid nitrogen and stored at -70°C until used for RNA isolation.

Depilation experiments: C57/BL6xCF-1 mice were used for the depilation experiments so that the stage of hair follicle growth could be monitored (Paus et al., 1990). For depilation experiments, adult mice (6-8 weeks) were anesthetized with Avertin and the hair was removed using a mixture of bees' wax and rosin (ZIP; Lee Pharmaceuticals, South El Monte, CA). On the specified days after depilation (day 0), mice were sacrificed and each sample of depilated skin was cut in half for analysis by immunohistochemistry and RT-PCR respectively.

Immunohistochemistry: Animals were killed on days 0 through 10 after wounding or 2.5 through 17.5 days after depilation. Tissues were immediately
fixed in 4% paraformaldehyde in PBS (0.14 M NaCl, 2.7 mM KCl, 4 mM Na₂HPO₄, 14.7 mM KH₂PO₄, pH 7.4) for 1-2 h at 4°C. Samples were then rinsed in PBS and stored in 70% ethanol until sectioned. For immunodetection of MRP, 6-μm sections were rehydrated and stained as previously described (Allen and Nilsen-Hamilton, 1998) by using a polyclonal rabbit anti-MRP/PLF serum (89rb13a) or a pre-immune serum from the same animal, each at a dilution of 1/500. Primary antibody was detected using biotinylated goat antirabbit and horseradish peroxidase conjugated to avidin and was visualized by a peroxidase substrate, diamino benzidine tetrahydrochloride (ABC kit; Vectastain; Burlingame, CA). Samples were counter-stained with hematoxylin and eosin, dehydrated, and mounted with Permount (Fisher Scientific, Pittsburgh PA) with a coverslip on top.

**RNA isolation, RT-PCR, and Diagnostic RT-PCR** Frozen tissue was pulverized in liquid nitrogen using a mortar and pestle, and total RNA was isolated by using Tri-Reagent (Gibco BRL, Gathersburg, MD) according to the manufacturer's instructions. Reverse transcription and RT-PCR were performed as previously described (Fang *et al.*, 1999; Fassett *et al.*, 2000). For PCR of mrp/plf cDNAs, the following primers were used; DE1;

5'TAAGCCTGGGTAGGACTCTGCA 3' (+42 to +63), UEV;

5'CATGATATTTCCAGAAGCGAGGAC3' (+776 to +754). For G3DPH, the primers used were 5'TGTGGATGGCCCTCTGGAAA3' (+601 to +621) for the upstream primer and
5'-GTTTCTTACTCCTTGAGGC3' (+1053 to +1034) for the downstream primer.

The diagnostic RT-PCR assay for the mrp/plfs is based on minor differences in the cDNA sequences of these closely related gene products that result in the presence or absence of restriction sites within the cDNA (Fang et al., 1999; Fassett et al., 2000). Amplified, radiolabeled fragments were digested with 1 unit each of BsoFI (New England Biolabs; Beverly, MA) and BstXI (New England Biolabs) at 55°C overnight in 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 100 µg/ml bovine serum albumin, 10 mM Tris-Cl (pH 7.9 at 25°C). The radiolabeled and digested products were resolved by electrophoresis through 10% non-denaturing polyacrylamide gels. The gels were dried and exposed to film. Positive controls for identification of specific mrp/plfs were plf1, plf2, mrp3 and mrp4 cDNAs.

**Keratinocyte isolation and culture** Newborn mouse keratinocytes were isolated as previously described (Harper et al., 1988) but with some minor modifications. Briefly, newborn (day 3-4) mouse skin was removed and incubated overnight in 0.25% trypsin in Hanks balanced salts without Ca²⁺ or Mg²⁺ (HBS; Gibco BRL). Epidermis was separated from dermis with tweezers, and then the dermis was gently scraped with a razor blade to remove remaining keratinocytes which were included with the epidermis. Epidermis was finely minced in Dulbecco-Vogt's modified Eagles medium with high glucose (Gibco BRL) and containing 10% calf serum. Larger pieces were removed with a
tweezers. The cells were collected by centrifugation at 500 x g for 10 min, washed in HBS, and resuspended in serum-free keratinocyte media (Gibco BRL) containing 25 μg/ml bovine pituitary extract (Gibco BRL), 2.5 ng/ml recombinant EGF (Gibco BRL), 100 units/ml Penn/strep, and 90 μM CaCl$_2$. After being cultured overnight unattached cells were removed and the medium was replaced with fresh medium as before but with 75 μM CaCl$_2$. Keratinocytes were cultured for up to four days before the medium was changed and then the cells were cultured for an additional two days without changing the medium. The medium was removed and fresh serum-free medium was added without pituitary extract, but with the indicated growth factors. Total RNA was isolated after an additional 14-17 h of culture.

**Transgenic mice** Transgenic mice were produced by microinjection of a linearized construct as described by others (Hogan et al., 1994). The full-length construct (CMV/mrp3/sisGal/3U) contains the following elements in order of 5' to 3': A 307 bp Nru1-Ban1 fragment containing the CMV immediate early enhancer from pcDNA-3 (Invitrogen), a 1514 bp Xba1 to Pst1 fragment of the $mrp3$ promoter (-1450 to +64), the adenovirus/IgG hybrid intron (Choi et al., 1991), the lacZ gene, a PvuII/EcoRI fragment including part of exon five of the $mrp3$ gene to and the 3' poly A site (170 bp) and about 600bp 3' to the PvuII/EcoRI fragment in the $mrp3$ gene. As well, the full-length construct contained the adjacent ~3,000 bp EcoRI fragment that is 3' to the PvuII/EcoRI 3' end of the $mrp3$ gene.
Partial constructs were also used to prepare transgenic animals. These partial constructs consisted of the same portions of the transgene just described, but were lacking either the 3kb EcoRI fragment of the mrp3 gene or the 5' mrp3 sequence from -42 to -1450 of the mrp3 promoter and have ~600 bp instead of ~3kb of the 3' mrp3 UTR. To prepare the construct for microinjection, the DNA was digested by Not I to remove it from the vector (pckt17-2), resolved by electrophoresis through a 0.8% agarose gel, and purified with Gene Clean 101 (Bio 101; La Jolla, CA). The eluted DNA was precipitated with ethanol, washed twice in 70% ethanol, and resuspended in 150 μM EDTA, 10 mM Tris·HCl, pH 7.4.

Transgenic mice were identified by PCR analysis of DNA isolated from tail or ear tissue. β-galactosidase activity in wounds or hair follicles was detected as follows. Tissues were fixed for 40 min in 4% paraformaldehyde in PBS then washed 3 times in PBS containing 0.01% Tween 20 and 0.02% sodium deoxycholate. The tissue was then stained overnight at 30°C in 0.01% Tween 20, 0.02% sodium deoxycholate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 1 mg/ml X-gal, and 0.1 M sodium phosphate, pH 7.4.

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REFERENCES


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FIGURES

LEGENDS TO FIGURES

Figure 1. MRP/PLF localization in skin wounds. Immunohistochemistry of day 5 skin wounds by using anti-MRP/PLF antisera (A) or preimmune serum (B) demonstrates MRP staining localized to keratinocytes at the edge of the wound.

A, inset. In an expanded view of a section of A, staining is seen to be most evident in the suprabasal layer of the epidermis, less evident in the stratum granulosum, and not evident in the basal layer. BA basal layer; CR, crust; HF, hair follicle; SC, stratum corneum; SG, stratum granulosum. Arrows in panel A point to sections stained by the antibody. The long vertical arrow in panel B shows the centre of the wound.

Figure 2. Mrp/plf RNA is expressed during wound healing and the major form of mrp/plf expressed during wound healing is mrp3. A. RT-PCR performed on total RNA demonstrates that mrp/plf mRNA is expressed between days 1 and 8 after wounding (top panel). Amplification of GD3PH was similar in all samples (middle panel), while no signal was detected in the absence of reverse transcriptase (bottom panel). B. To determine which mrp/plfs are expressed during wound healing, portions of the amplified mrp/plf cDNAs were amplified for one additional round in the presence of $^{32}$P-dCTP, digested with Bsof1 and Bstx1, and separated on a nondenaturing 10% polyacrylamide gel. Samples of the four cloned mrp/plfs (1 through 4) were similarly treated and serve as controls to identify the expected fragments. Different fragments result
from the digestion of \textit{plf1} (318, 246, and 110 bp), \textit{plf2} (289, 275, and 110 bp), \textit{mrp3} (289, 246, and 110 bp), and \textit{mrp4} (564 and 110 bp). Shown are samples from two samples of day 2.5 wounds, each removed from a different mouse.

Figure 3. \textbf{Mrp/plf3 expression is induced by KGF} Newborn mouse keratinocytes were cultured and treated with growth factors as described in Materials and Methods. Concentrations of individual growth factors were each 10 ng/ml. \textit{Mrp/plf} mRNAs were detected by Northern blot analysis. EGF and TGF\textalpha did not induce expression of \textit{mrp/plfs}, and KGF caused a 6-fold induction relative to untreated keratinocytes. A. Quantitative results from Northern blot in which the value for \textit{mrp/plf} mRNA level was normalized to the level of G3PDH mRNA in each lane. Samples were in duplicate. B. Northern blots comparing the effects of EGF and KGF on keratinocyte mRNA levels. C. Diagnostic RT-PCR shows that cultured keratinocytes express mostly \textit{mrp3} compared with 3T3 cells, which produce mostly \textit{plf1}.

Figure 4. The MRP3 promoter directs appropriate expression of \textit{lacZ} in healing wounds Three to four days after wounding, expression of the CMV/mrp3/lacZ/3u transgene is detected in cutaneous wounds in the back (A) or in tail wounds (B) and (C). Shown is a 4-day old back wound of about 1 cm in length (A), 3-day-old tail wound (B) and a second more proximal cut in the same tail as in panel B, but which was fixed immediately after wounding (C). Arrows point to locations of blue stain.
Figure 5. Expression of MRP/PLF in the Hair Follicle

The outer root sheath of anagen-stage hair follicles were immunostained for MRP/PLF. The section shown is a region of skin in an FVB female mouse in which many hair follicles were stained. Serial sections were stained with anti-MRP/PLF (A), or pre-immune serum (B). C57/BL6xCF-1 mice were depilated and sacrificed 2.5 (C,D), 8 (E,F), and 17.5 (G,H) days later. Skin samples were removed, fixed and immunostained with anti-MRP/PLF (D,F,H), or pre-immune serum (C,E,G). Arrows point to locations of red-brown stain. The center of the hair-shafts in the C57/BL6xCF-1 mice also show as dark because of the high concentration of melanin pigment in these hair shafts.

Figure 6. Mrp/plf mRNA Levels Vary During the Depilation-Induced Hair Cycle

Total RNA was isolated from depilated skin 0, 2.5, 8, or 17.5 days after depilation. Duplicate samples are shown, each from a different animal. A. RT-PCR was performed as described in Materials and Methods, using primers for mrp/plf or g3pdh. B. Portions of the day 17.5 samples shown in panel A were analyzed by diagnostic RT-PCR as described in Materials and Methods. The duplicate samples are each from a different animal. Mrp3 is the major mrp/plf expressed during the hair follicle cycle although plf1 was also detected.

Figure 7. The mrp3 Promoter Directs Expression of LacZ to the Outer Root Sheath During the Hair Cycle

CMV/MRP3/lacZ/3U transgenic mice were shaved then depilated. The tissue was fixed, sectioned and stained for β-galactosidase activity (A). Hair follicles were also plucked, fixed and stained for
β-galactosidase activity (B). Transgene expression appears in the same location as the immunostain of endogenous MRP/PLF was found in other tissues slices (A) and can be seen in similar locations in the plucked hair follicles (B).
Figure 2
Figure 3
Figure 4
Figure 6
CHAPTER 5: TISSUE SPECIFIC SPlicing AND EXPRESSION OF AN
MRP/PLF3 TRANSGENE IN THE PRESENCE OF A UBQUITOUS ENHANCER
IS MEDIATED BY FUNCTIONAL VERSUS DECOY PROMOTER SELECTION

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Abstract

The m/r/p/lf genes are expressed specifically in the trophoblastic giant cells at high levels during midgestation, and are also expressed in some immortalized cell lines in response to growth factors. In cell lines, m/r/p/lf induction is reported to occur through transcriptional and post-transcriptional mechanisms. To investigate the mechanism by which m/r/p/lf genes are expressed in a tissue specific fashion in vivo, the lacZ gene and an adenovirus/IgG hybrid intron were inserted between 1.5 kb of m/r/p/lf3 promoter and 3kb of 3' sequence. This construct was unable to drive detectable β-galactosidase activity in transgenic placentae, but was active in transient transfections. If the hCMV enhancer was

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added to the 5' end of the transgene, giant cell specific transgene expression was observed, suggesting that the mrp3 sequences in the construct can direct tissue specific expression in the presence of a ubiquitous enhancer. Removal of the 3' 3kb region increased non-specific expression in transgenic embryos, and removal of 5' mrp3 sequences resulted in more aberrant expression. It is further demonstrated that the mrp3 transgene contains alternate upstream promoters that drive transcription of nonsense containing transcripts that may either be degraded after splicing or not efficiently spliced. Endogenous mrp/plf transcripts from these upstream promoters are also produced in vivo. When upstream promoters of the mrp3 gene are removed, expression of a reporter gene increases in transient transfection, as does the level of spliced transcripts. The ratios of spliced to unspliced transcripts also increases in transgenic mouse tissues or cells in conditions that promote mrp3 expression. This suggests a model by which tissue specific expression of the mrp3 gene may be obtained in the presence of a strong ubiquitous enhancer. That is, giant cell specific factors bound to mrp3 cis acting elements influence selection of the correct promoter for activation by the CMV enhancer, while non-specific expression is repressed in absence of these factors by selection of decoy promoters that do not result in functional transcripts. This model of gene regulation could serve as a fine tuning mechanism for tissue specific or growth factor inducible expression in the presence of ubiquitous activators of transcription, and could account for what
appears to be post-transcriptional regulation of endogenous mrp/plf genes or other genes which demonstrate nuclear post-transcriptional regulation.

Introduction

Recently, it has become evident that events associated with transcriptional regulation of gene expression may also effect post-transcriptional processing of RNA. For instance, the use of different promoters can effect the processing of the same downstream transcript and phosphorylation of the carboxy terminus domain (CTD) of RNA polymerase II is required for efficient splicing (Misteli and Spector, 1999; Steinmetz, 1997). Phosphorylation of the CTD is also necessary for recruitment of the protein complex required for addition of the 5' cap of newly transcribed RNA (Cho et al., 1997; McCracken et al., 1997). It is thus possible that differences observed in post-transcriptional regulation of a gene may be related to interactions of RNA polymerase with transcription factors that could even occur prior to initiation of transcription. Because as much as 1/3 of all exon-containing RNA is retained and degraded in the nucleus (Jackson et al., 2000), understanding how transcription and nuclear processing may be connected is critical for truly understanding mechanisms of tissue specific gene regulation. One of many genes that demonstrates highly tissue specific expression in the mouse, and also demonstrates transcriptional and nuclear post-transcriptional regulation in cultured cells, is the mitogen regulated protein gene (proliferin; mrp/plf).
Mitogen Regulated Protein (proliferin; *mrp/plf*) was originally identified as a secondary response gene that is up-regulated in 3T3 cells by serum or growth factors (Nilsen-Hamilton et al., 1980; Linzer and Nathans, 1984; Nilsen-Hamilton et al., 1987). There are between four and six *mrp/plf* genes in mice (Jackson-Grusby et al., 1988; Wilder and Linzer, 1986) and promoters have been isolated from three of them (Connor *et al.*, 1989; Linzer and Mordacq, 1987). In transfected 3T3 cells, *mrp3* promoter/reporter constructs are activated by basic fibroblast growth factor (bFGF; Mohideen *et al.*, 1999), and in L-cells, *plf1* promoter/reporter constructs respond to TPA or serum stimulation (Linzer and Mordacq, 1987; Mordacq and Linzer, 1989). However, endogenous 3T3 *plf1* message increases in response to bFGF or serum without apparent differences in transcription as measured by the nuclear run-off assay (Mohideen; thesis, Iowa State University; Linzer and Wilder, 1987). Similarly, there is no difference in transcription levels between 3T3 cells, that express high levels of *mrp/plfs* and primary mouse embryo fibroblasts, that don’t express *mrp/plf* and from which 3T3 cells are derived (Edwards *et al.*, 1987; Malyankar *et al.*, 1994). In both cases, higher expression levels appear to be related to post-transcriptional processing and increased stability of transcripts in the nucleus (Mohideen thesis; Edwards *et al.*, 1987; Malyankar *et al.*, 1994).

During development, at least three *mrp/plf* genes (*plf1*, *mrp3*, and *mrp4*) (Wilder and Linzer, 1986; Fang *et al.*, 1999; Fassett *et al.*, 2000) are expressed
at high levels in the trophoblastic giant cells of the placenta (Lee et al., 1988; Nilsen-Hamilton et al., 1988), where they are believed to act as angiogenesis factors and uterine growth factors (Jackson et al., 1994; Nelson et al., 1995). Of these genes, mrp3 is the most highly expressed, with peak levels occurring between days 9 and 11 (Fang et al., 1999; Fassett et al., 2000). We have recently determined that mrp/plf expression is also detectable in some adult mouse tissues under certain conditions (Fassett et al., 2000). Mrp3 expression is induced in keratinocytes of the adult mouse during wound healing and the hair follicle cycle (Fassett and Nilsen-Hamilton; submitted), plf1 is expressed in the epithelia of the stomach (Fassett, unpublished) and small intestine (Fassett et al., 2000), and mrp4 is expressed in the mouse ear and tail (Fassett et al., 2000).

While a number of reports have identified serum or growth factor-responsive elements in the mrp/plf promoters, there have been no reports demonstrating how mrp/plf gene expression is directed specifically to the placenta or other tissues. Based upon studies in cultured cells, it is likely that mrp/plf genes are regulated by both transcriptional and post-transcriptional processing mechanisms in vivo.

To better understand how tissue specific expression of the mrp/plf genes is regulated in vivo, 1514 b.p. of 5' sequences and 3600 b.p. of 3' flanking sequences of the mrp3 gene were analyzed in transgenic embryos for the ability to direct expression of a hybrid intron/lacZ reporter gene. Our results
demonstrate that the sequences included in the mrp3 transgene alone lack the elements necessary to direct detectable expression in transgenic embryos, however, giant cell and also wound and hair follicle specific transgene expression is demonstrated if a strong heterologous enhancer (hCMV.IE) is included at the 5' end of the gene. These observations suggest that the 5' and 3' flanking regions of the mrp3 gene are able to mediate tissue specificity under the influence of a fairly ubiquitous and constitutive enhancer. Importantly, it is also demonstrated here that the 5' flanking sequences of the mrp3 gene affect splicing of the hybrid intron present in the transgene in a tissue specific fashion. Further characterization of this effect suggests that unspliced transcripts are derived from transcriptional start sites upstream of the commonly defined promoter and they contain translational start codons followed by nonsense codons. Transcripts beginning upstream of the normal start site are also produced by the endogenous mrp/plf genes in mice and in 3T3 cells and are differently processed. These results couple selection of the correct transcript initiation site to efficient post-transcriptional processing, and suggest a cryptic fine tuning mechanism by which proximal flanking sequences may control gene expression even in the presence of a strong constitutive enhancer.

Materials and Methods

Plasmids The construct (CMV/1514mrp3/sisGal/3U) contains the following elements in order of 5' to 3'; A 507 bp Nru1-BanI fragment containing the CMV
immediate early enhancer from pcDNA-3 (Invitrogen; Carlsbad, CA), a 1514 bp Xba1 to Pst1 fragment of the *mrp3* promoter (-1450 to +64), the adenovirus/IgG hybrid intron (Huang and Gorman, 1990), the lacZ gene, 170 bp of exon five of the *mrp3* gene to and including the poly A site, and the adjacent 3600 basepairs of the *mrp3* gene. Construction of the CMV/1514mrp3/sisGal/3U plasmid containing the full-length *mrp3* gene was previously described (Fang *et al*., 1999). The construct 1514mrp3/sisGal/3U is the same as above, but missing the CMV enhancer. CMV/1514mrp3/sisGal/0.6U is the same as CMV/1514mrp3/sisGal/3U, but is missing a 3000 basepair EcoRI fragment of *mrp3* 3' untranslated region. Partial constructs also used for making transgenic animals or in cell culture include the CMV(-49)mrp3/sigal/0.6U construct, produced by digesting with Bsu36I and XbaI to remove the *mrp3* promoter sequences upstream of -48 from CMV/1514mrp3/sigal/0.6U. The CMV(-230)mrp3/sigal/0.6U and CMV(-130)mrp3/sigal/0.6U constructs were produced using PCR to amplify fragments between -230 or B130, and the hybrid intron, which were then digested with SacII and SpeI (a SpeI site was inserted into 5' end of fragment as part of the primer) and cloned in place of the 1514 basepair *mrp3* flanking sequence at the SacII and XbaI sites from which the 1514 basepair sequence was released. The plasmid SVisiscat, which expresses the hybrid intron/Choramphenicol transferase gene under control of the SV40 promoter was kindly provided by Cornelia Gorman.
Transgenic Mice Transgenic mice were produced by pronuclear microinjection as previously described (Hogan). To prepare the construct for microinjection, the DNA was digested by Not I to remove it from the vector (pckt17-2), resolved by electrophoresis through a 0.8% agarose gel, and purified with Gene Clean 101 (Bio 101; La Jolla, CA). Eluted DNA was precipitated with 0.3M Na Acetate pH 7 and two volumes 100% ethanol, washed twice in 70% ethanol, and resuspended in 150 μM EDTA, 10 mM Tris, pH 7.4. F0 embryos were dissected from the uterus and part of the yolk sac or 1/2 the placenta was removed for analysis of DNA or RNA respectively. Transgenic embryos were identified by PCR on the yolk sacs as previously described (Biotechniques ref*). Embryos identified as positive by PCR but which did not produce visible β-galactosidase activity were further analyzed by junctional PCR across transgene ends to exclude the possibility of contamination by plasmid DNA. To visualize in situ β-galactosidase activity, embryos or adult tissues were fixed by rocking in 4% paraformaldehyde in PBS pH7.4 for 40 minutes at 4°C, washed three times in PBS pH 7.4 containing 0.1% NP40 and 0.2% deoxycholate for 30 minutes each at 23°C, and then stained in 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM MgCl₂, 0.1%NP40, 0.2% deoxycholate, and 1mg/ml X-gal in 0.1M sodium phosphate, pH 7.4 at 32°C.

Transfection and cell culture COS1 cells were grown in DME with high glucose (DMEG) with 10 % calf serum and transfected using the DEAE
dextran/chloroquine method as previously described (Kingston, 1987). Total RNA was isolated using Trizol reagent as described by the manufacturer (Gibco BRL; Gathersburg, MD). 3T3 cells were cultured in DME with pyruvate (DMEP) and 10% calf serum. To stimulate cells with bFGF, cells were plated out at 50% confluency, incubated for 48 h at 37°C with 10% CO2, then bFGF was added at 10ng/ml for an additional 14 to 17 h.

**RT-PCR** For RT-PCR, DNA was first removed from 5µg RNA using 100 units RNase free DNase (Gibco BRL) in a 50 µl sample containing 10µg glycogen, 40 units RNASin, 5mM MnCl2, 10mM Tris-HCl, pH 8.0, for 40 minutes at 37°C. Samples were extracted twice with phenol chloroform (25:24:1), pH 8.0, precipitated with 0.3M sodium acetate, and two volumes 100% ethanol, and washed once in 70% ethanol. The samples were allowed to dry and then resuspended in 20 µl of superscript reverse transcriptase buffer containing 0.5 mM dNTPs and 250 ng of random primers, 3 mM MgCl2, 10 mM DTT, 75 mM KCl, in 50 mM Tris-HCl (pH 8.3 at 23°C). Each sample was then divided into two 10 µl portions and heated to 55°C for 5 min. One multiple of each sample received 0.5 µl (20 units) superscript reverse transcriptase (Gibco BRL). Samples were reverse transcribed for 10 min at room temperature, 1 h at 37°C, 1 h at 42°C, 10 min at 50°C, and then 15 min at 75°C.

For amplification of transfected or transgenic cDNAs, the following primers were used: 1) for lacZ, ULAC; 5’ aactgttgtggaagggcatcg3’ (+129 to +108 from
lac Z start codon) For mrp1/plfls; NUE5 5'catgatattcagaagcagacgac3'(+776 to +754), UE3 5' agaagtattgcatctcatgggg 3'(+336 to +315), DAP1; 5' ccactagtcagagcataaacct 3' (-229 to -213), M3V55; 5' ccactagtcagagcatgctggttctatggctactt 3'(-129 to -110), D180; 5' taacacgctctttctctcttg 3' (-83 to -63), and D4263; 5' gctaagcctggtaggactct 3' (+42 to +63). Primers used for the hybrid intron and the CAT gene were dsis 5' cccttctagaattcgctgtctgc 3', and UCAT 5' cagcacctgtctgtcctgtcgc 3' respectively. PCR was performed in 25 µl reactions containing 0.2 mM dNTPs, 1.5 mM MgCl₂, 10 pmoles of each primer in 50 mM KCl, 10 mM Tris-HCl, pH 8.3. The PCR cycles were: one cycle at 95°C for 30 sec, one cycle at 72°C for 2 min during which time Taq polymerase was added. The DNA was then amplified with 32-40 cycles at 95°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, with one final extension at 72°C for 5 min. Samples were separated on 1.5 % agarose gels. RACE; Primers used for RACE are UE3; 5' agaagtattgcatctcatgggg 3'(+336 to +315), Uil; 5' agacactgctgcatgactctagg3' (+260 to +238 of intron 1), danch; 5' ggccacgcgtcgactgtacgctagc3', and Anchor primer; 5' ggccacgcgtcgactgtacgctagcggggiig3' (Gibco BRL). 0.2ug RNA was reverse transcribed in 50 mM Tris·Cl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, 10 mM DTT with 0.20 mM dNTPs, and 5 pmoles primer ue3 or uil and 4 units Rnasin + or - 2.5 units reverse transcriptase. Samples heated for 3 min at 55°C then put on ice and 0.5 ul AMV RT was added (2.5 units) while the
random primed samples stayed at 37°C. cDNA samples were treated with 1 μl RNase H for 10 minutes at 37°C, then DNA was purified using gene clean (Bio 101) and 5 μl glassmilk. Samples were resuspended in 10 μl water and 7 μl was used for c-tailing in a 10 μl reaction containing 100 mM cacodylate buffer (pH 6.8), 1 mM CoCl₂, 0.1 mM DTT, four units terminal deoxynucleotidyl transferase (tdt) and incubated at 37°C for 5 min. and 65°C for 5 min. Samples were then diluted with 100 μl TE pH 8.0. For PCR, 0.5 μl of c-tailed cDNA were used in a 12.5 μl reaction containing 10 mM Tris-HCl (pH 9 at 25°C), 50 mM KCl, 0.1% Triton x-100, 0.2 mM dNTPs, using 10 pmol anchor primer (gibco BRL), and 10 pmol primer UE1 with the following cycles. 1 cycle of 95°C for 30s, 52°C for 2 min. 72°C for 10 minutes. Then 35 cycles of 95°C for 30s, 55°C for 45s, 72°C for 90s, then a 5 min extension at 72°C. A second round of PCR was performed in a 12.5 μl reaction using 0.5 μl of sample from the first round of PCR and 10 pmol primers UE1 and d-anchor, (specific to the 5' end of the anchor primer). 30 cycles of PCR were performed at 95°C for 30s, 65°C for 30s, and 72°C for 60s, followed by a 5 min extension at 72°C.

Results

The mrp/plf3 gene requires an enhancer in vivo

The mrp/plf promoters from -1100 to +64 have been used previously to drive growth factor or serum regulated expression of CAT reporter genes in
transfected cells (Linzer and Mordacq, 1987; Mohideen et al., 1999). To analyze activity of the *mrp3* regulatory elements in vivo, a hybrid intron composed of the adenovirus splice donor and IgG splice acceptor (Huang and Gorman, 1990) and the lacZ gene were inserted between 1514 basepairs of 5' *mrp3* flanking sequence (-1450 to +64), and the fifth exon of *mrp3* (+660 to the poly A signal at +790) with approximately 3600 basepairs of 3' flanking sequence downstream to produce the plasmid construct -1450mrp3/sis/lac/3.6. These transgenes produced no detectable reporter gene activity when integrated into the genome of mid-gestational embryos (Table 1), yet are active in transient lipofection mediated transfections of cultured COS cells, BNL cells, or trophoblastic giant cells (not shown). It was thus possible that the transgene was being inactivated when integrated into chromatin, and might be missing an enhancer needed to overcome inhibitory chromatin structure. To test the hypothesis that this construct could be active when integrated into chromatin in vivo when a strong enhancer was present, the -1450mrp3/sislac/3.6 construct was co-injected with a β-actin driven expression cassette containing 5kb of 5' flanking sequence and 800 basepairs 3' untranslated sequence. This cassette expresses β-galactosidase ubiquitously in mid-gestation embryos (not shown). The lacZ gene was replaced in this construct with the *plf1* cDNA. Co-injection with the ubiquitously active β-actin cassette resulted in detectable β-galactosidase activity in 2 out of 5 transgenic embryos, but expression was ubiquitous in one
embryo/placenta, and scattered throughout many different cell types in the other. This suggests that co-integration with an active gene allowed the previously inactive \textit{mrp3} transgene to be active. Further work was not done however to rule out the effects of \textit{pltf1} overexpression, the possibility of recombination between sequences present in both transgenes, or the possibility that transcription from the actin promoter was reading through to the \textit{mrp3} gene and splicing was occurring between the actin intron and the hybrid intron in the \textit{lac Z} gene. To further investigate the effect of a strong enhancer upon \textit{mrp3} promoter activity, but to avoid the complications discussed above, the hCMV immediate early enhancer was added to the 5' end of the construct, so that only the \textit{mrp3} promoter would be available to the enhancer (CMV/-1450mrp3/sislac/3.6). When tested in e11 transgenic embryos, the spatial expression of this construct paralleled endogenous expression of \textit{mrp/pltfs}, showing \textbeta-galactosidase activity only in the trophoblastic giant cells of the placenta (Fig 1 and table 1). At e13, three of four embryos displayed \textbeta-galactosidase activity in the embryo proper, but still displayed giant cell specific expression in the placenta (not shown). In most of the transgenic embryos, expression was not evident in the majority of giant cells, but appeared in a variegated pattern, and at times was limited to only a few giant cells. In one of two lines produced with this construct, the transgene also expressed in adult mice in a pattern similar to endogenous \textit{mrp3}, demonstrating \textbeta-galactosidase activity induced during cutaneous wound healing.
and during the hair follicle cycle (Fassett; submitted). Ectopic expression was observed however in the liver, but was absent from kidneys, lungs, stomach, spleen, heart, and testes. The second transgenic line prepared with this construct demonstrated lower levels of \( \beta \)-galactosidase activity but expression was still detectable in tail wounds. F1 offspring of the transgenic adults also expressed the transgene in giant cells, but in a small number of giant cells and in a variegated pattern, similar to the majority of the F0 transgenics. The transgene was also expressed in the decidua of pregnant female transgenic mice at high levels (not shown). At this time, it is not clear whether this represents ectopic expression or specific expression, as \( mrp/pfl \) RNA is detectable by Northern blot from this tissue (Linzer et al., 1985), and protein is also evident in the decidua using immunohistochemistry (unpublished), but in situ hybridization failed to detect the \( mrp/pfl \) signal in the decidua relative to extremely high levels of giant cell expression (Lee et al., 1988).

**Mrp3 5' and 3' flanking sequences mediate tissue specific expression under the influence of the CMV enhancer**

The CMV enhancer is active in numerous tissues of the midgestational embryo and the adult mouse, but not in the midgestational placenta (Baskar et al., 1996a; Baskar et al., 1996b, Koedood et al., 1995). Our results showing that inclusion of the CMV enhancer upstream of the \( mrp3 \) promoter sequences
results in specific expression in the placental giant cells suggests that elements within the *mrp3* promoter and surrounding sequences are able to repress expression in most tissues and/or these elements interact with the CMV enhancer to induce transgene expression specifically in the trophoblastic giant cells.

To identify sequences that may act by either suppressing non-specific expression or inducing tissue specific expression, a series of deletion mutants were examined either in transfected cells or in transgenic embryos. Deletion of 3kb of *mrp3* sequences from the 3' end (CMV/1514mrp3/sis/gal/0.6U) still resulted in giant cell expression, but caused a higher level of non-specific expression in e11 transgenic embryos. The expression patterns of β-galactosidase activity in these embryos and placentae varied dramatically, suggesting that the site of integration might also effect the activity of the transgene (table 1). In two lines of transgenic mice containing this construct, embryonic expression also varied, with one line expressing in both the giant cells and the embryo proper, and the other demonstrating very little expression in the embryo or placenta.

To investigate the role of the *mrp3* 5' flanking sequences in tissue specific expression, a further deletion of the 5' end of the *mrp3* gene was made, so that only 113 bp of *mrp3* upstream sequence remained (-49 to +64). The resulting animals displayed more aberrant expression and less staining in giant cells than
animals expressing from the longer mrp3 promoter sequences, suggesting giant cell specificity is also regulated by elements between -49 and -1450. Four out of the 6 transgenic embryos produced with this construct were less developed than 12 non-transgenic animals produced in the same experiment. A single line was produced with this construct, in which high level expression was evident in all embryonic and adult tissues examined. Figure 2 demonstrates that while the full length mrp3 transgene is only expressed in response to wound healing or during the hair follicle cycle, the minimal (-49 bp) mrp3 construct was expressed throughout all tissue layers of the tail. This construct also expressed β-galactosidase activity by the 16 cell stage of development, while the full length construct appears inactive at this time point (data not shown).

Hybrid intron not efficiently spliced in transgenic placentae

In some cases, one half of each placenta of the F0 embryos was saved for RNA or DNA analysis. RT-PCR was performed on samples using primers that amplify from the 5' untranslated region of the mrp3 gene across the hybrid intron to the lacZ gene. In most transgenic samples lacking an enhancer, no lacZ RNA was detected, however, in two that were, very little splicing of the transgene was observed. As well, splicing was inefficient in a placenta from a embryo produced using the -1450sislac3600 construct co-injected with B-actin cassette, and also from a transgenic placental sample containing the cmv/-1450sislc600 transgene.
(Fig 3). This intron is completely spliced when under control of the SV40 promoter in transfected cultured cells (not shown).

**Tissue/cell specific splicing of hybrid intron**

To further investigate splicing of the transgene, we examined other conditions where *mrp3* expression is induced. Recently we have detected *mrp3* expression in the hair follicles and also during wound healing and in isolated keratinocytes (Fassett and Nilsen-Hamilton, submitted). In the skin of a 13 day old mouse, which contained numerous β-galactosidase positive hair follicles, there was a higher level of spliced relative to unspliced transgene, than in the heart of the same mouse, and where transgene expression is not detectable (figure 4). In isolated 4-day old mouse keratinocytes, the cell type that expresses *mrp3* in the skin, there was a much higher level of splicing than in whole skin samples from adults of the same line. KGF, which induces *mrp3* expression in keratinocytes, had little effect on the level of splicing, suggesting that tissue specific splicing is a function of the cell type and not a consequence of an external signal such as from a growth factor.

RNA from a skin wound, where the transgene is induced, appeared to be spliced at a higher relative level than skin from a non-wounded transgenic mouse of the same line. Another band smaller than the predicted size of spliced transgene also appeared which has not been characterized. Skin from the line
of transgenic mouse containing the minimal promoter downstream of the CMV enhancer (CMV/(-49)sisl/lac/0.6U) however, contained mostly spliced transgene transcripts. Together, these results suggest that splicing levels are higher in the cells or tissues that express mrp3 (keratinocytes). Other cell types besides keratinocytes within the whole skin or in tissues such as the heart, which do not express mrp3, may contribute to a higher level of unspliced transcripts within the total RNA population. Furthermore, the complete splicing of the transcribed transgene containing the minimal mrp3 promoter suggests that sequences upstream of position -49 may contribute to a lower level of splicing and transgene expression in tissues that don't express mrp3.

One mechanism by which upstream 5' flanking sequences of the mrp3 gene could effect splicing is by acting as alternate transcript initiation sites that result in the production of nonsense transcripts. Nonsense mutations have been shown to inhibit splicing (Aoufouchi et al., 1996), or result in altered nuclear stability or processing (Hentze and Kulozik, 1999; Maquat, 1995). Because the 5' region of the mrp3 gene appears to effect splicing of a heterologous intron, and the endogenous plf1 transcript level is regulated by post-transcriptional RNA stability and RNA processing in 3T3 cells, we analyzed 3T3 cells, placentae and embryo for the presence of upstream nonsense transcript producing promoters.
Upstream alternative transcript initiation sites in endogenous *mrf/plf* genes are differently processed

RACE was used to map the transcript initiation sites in e11 embryo nuclei and unstimulated 3T3 nuclei, where *mrf/plf* expression is absent or low, or in e11 placentae or FGF stimulated 3T3 nuclei in which *mrf/plf* expression is high. Initial reverse transcription was performed using primers that hybridize to intron I (UI1) or exon III (UE3), while the final round of amplification used sequential primers which hybridize to exon I so that the transcription start site could be mapped for unspliced versus spliced transcripts in these conditions. E11 embryo nuclei did not produce any detectable transcript using either primer. Using either initial primer, the majority of the PCR products of the placenta demonstrated the size of fragment predicted from amplification of a product between the poly Gl race primer, the normal transcriptional start site and the UE1 primer. By contrast, the 3T3 cell transcripts contained a larger sized PCR product when using UE3 compared with the UI1 for reverse transcription. The larger sized product appeared to increase in the absence of FGF, but was absent from samples reverse transcribed with primer UI1 (Fig 5a). The longer product produced from 3T3 mRNA was sequenced and found to start 142 bp upstream of the previously described start site for *plf1* (Linzer and Wilder, 1987).

To further investigate the processing of transcripts initiating from upstream
promoters, RT-PCR was performed on RNA samples using primers specific for intron 4 or exon 5, in combination with primers specific for either +42 to +63, or -82 to -62 (Fig. 6). Amplification of cDNAs from within +42 of the normal start site to 305 bp of intron 4, or +776 in exon 5, was observed in stimulated or unstimulated 3T3 cell nuclei and total placental RNA. cDNA derived from upstream initiation sites which contained intron 4 however, was only detected in the unstimulated 3T3 cells (Fig. 5b). In COS cells, a full length mrp3 gene containing all four introns, and under the control of the CMV(mrp3/-1514) enhancer sequence produced fully spliced transcripts derived from sites upstream of -82 or +63, but little incompletely processed transcript was observed. To further investigate the processing of transcripts beginning at endogenous upstream promoters, RT-PCR was performed on cytoplasmic or nuclear RNA isolated from bFGF stimulated or unstimulated 3T3 cells. As demonstrated in figure 5c, the cytoplasmic levels of transcripts derived from upstream start sites appears greater than the nuclear levels, whereas primers amplifying transcripts from the normal and upstream start sites appear at similar levels in the cytoplasm, suggesting that the upstream transcripts are either less stable in the nucleus or are transported to the cytoplasm more rapidly.
Upstream sequences repress functional expression and produce transcripts which are poorly spliced

To determine if upstream alternate promoter containing sequences could act as transcriptional repressors, two constructs that contained the CMV enhancer upstream of -130, or -231 were tested in transient transfected COS cells. The activities and splicing of these constructs as well as the CMV(-49) and the CMV(-1514) constructs were compared by measuring the levels of lacZ RNA compared to a cotransfected SV40 driven CAT vector (pSV40sis CAT). Both the CMV(mrp3) lacZ constructs, and the (pSV40sisCAT construct contained the same heterologous hybrid intron, so levels of lacZ RNA relative to cat RNA may be compared between the constructs using RT-PCR and allowing the CAT and lacZ DNA amplicons to compete for annealing of the common primer. The results in (Fig. 6) demonstrate that there was a higher level of LacZ RNA in the cultures transfected with the CMV(-49) and CMV(-130) construct relative to cat RNA than from transfections using the CMV(-1514) and CMV(-230). Significantly, the level of spliced to unspliced transcripts appeared higher in the CMV(-49) and CMV(-130) construct than the CMV(-1514) or CMV(-230) constructs (fig. 6). This suggests that upstream regions of the mrp3 gene, particularly between -130 and -230 contain repressors of activation by the CMV enhancer, and may inhibit splicing of the heterologous hybrid intron, or
specifically cause degradation of spliced lacZ RNA. It is also evident that the hybrid intron is completely spliced when placed downstream of the SV40 promoter in the SV40sisCAT construct, suggesting that the low level of spliced product is caused by the mrp3 5' flanking sequence and not by the intron itself. As well, if lacZ replaces the cat gene downstream of the hybrid intron (SV40sisLAC), the intron is also completely spliced. (Note: The spliced lacZ fragment is larger in this case because it is has an extended 5' untranslated region between the intron and the lacZ cosing region which adds approximately 200 basepairs.)

Analysis by RT-PCR demonstrates that transcripts originating upstream of the normal start site (+64) contain a higher ratio of unspliced to spliced intron than transcripts amplified from within the normal transcription start site. (fig 7). This suggests that upstream start sites are greater contributors to the total amount of unspliced RNA and supports the hypothesis that the transgene transcripts initiated at the correct promoter are more effectively spliced. Curiously, the construct containing the CMV/-130 mrp3 promoter produced mostly spliced RNA, but transcripts which began at or 5' of -130 were not spliced efficiently.

Discussion

The results presented illustrate two interesting aspects of gene regulation. First, tissue specific gene expression from an otherwise weak promoter is demonstrated in the presence of a strong, heterologous and somewhat
constitutive enhancer. Second, the 5' flanking sequences of the *mrp3* gene can effect splicing in a tissue or cell specific fashion, likely by modulating usage of either productive promoters or non-productive nonsense promoters.

The finding that 1514 bp of 5' flanking sequence of the *mrp3* gene and 3.6 kb of 3' flanking sequence was not sufficient to express detectable reporter gene activity in transgenic mice, suggested that other sequences were necessary for higher level expression. These missing sequences were in part substituted for by the heterologous hCMV IE enhancer. Because the hCMV IE enhancer can direct expression to the e11 embryo proper from it's own minimal promoter (Baskar *et al.*, 1996a; Koedood *et al.*, 1995) and in our constructs this occurs in the absence of 3' and 5' *mrp3* sequences, it suggests that *mrp3* sequences can repress activity of the CMV enhancer upon the *mrp3* promoter in the absence of tissue specific factors. The observation that the spatial expression of transgenes from the constructs lacking the 3' 3kb varied in each embryo examined suggests that downstream sequences possibly insulate the *mrp3* gene from the effects of surrounding sequences.

At this time it cannot be concluded whether repression occurs from specific elements in the flanking *mrp3* sequences, or by increasing the distance of the CMV enhancer, decreasing the chance of interaction with the proximal elements of the *mrp3* promoter, or from a combination of both of these effects. Proximity to the CMV enhancer has been shown to affect the level of expression from a
heterologous promoter (Kuhl et al., 1987).

It is likely that chromatin structure plays a large role in regulation of the mrp3 transgene and could be involved in regulation of the endogenous mrp/plf genes as well. This is suggested by the lack of detectable β-galactosidase expression from the mrp3 promoter in absence of an enhancer, the variegated expression pattern of the CMV/mrp3 transgene, and the widely varied pattern of expression observed between different F0 embryos in the transgenes containing deletions. Sensitivity to chromatin structure is also indicated by the observation that regulation by bFGF was only observed in 3T3 cells stably transfected with mrp3 promoter constructs (Mohideen et al., 1999).

Position effect variegation has been reported for expression of transgenes that are inserted into inactive chromatin (Boyer et al., 1997; Robertson et al., 1995). If the transgene is inhibited by chromatin structure, and because the CMV enhancer is not reported to be active in the placenta of midgestation embryos, it is possible that elements of the mrp3 gene interact with the CMV enhancer in a tissue specific fashion to increase the probability of overcoming inhibitory chromatin. Activation by an enhancer requires both the elements within the enhancer and an active promoter with which to interact. Removal of elements upstream of -49 from the mrp3 5' region produced β-galactosidase expression patterns which differed significantly from the expression pattern of the full length construct and the endogenous pattern of expression. Notably, with the exception of the single line of cmv-49/sis/lac/600 mice, expression in embryos did not
appear to be particularly strong, just more widespread and still variegated. In transient transfection, this construct appeared to express at much higher levels than the construct containing the -1450 sequence. Again this is suggestive of an interaction between flanking sequences of the mrp3 gene and the CMV enhancer to overcome chromatin structure, particularly in the giant cells. Elements that may be involved in regulating activity of the mrp3 promoter particularly in the trophoblastic giant cells could include GATA sites, a c-ets-2 element, or AP-2 element, all of which are found in the proximal mrp3 promoter and which bind proteins that have been demonstrated to be important for expression of placental specific genes or placental development (Ma et al., 1997; Shi and Kellems, 1998; Yamamoto et al., 1998). It is likely that a very strong enhancer or locus control region also regulates the endogenous mrp/plf genes, because endogenous levels of mrp/plf expression are extremely high in the giant cells during gestation. The related human growth hormone/human placental lactogen gene cluster, which is ~66 kb and contains five closely related genes, is regulated by a placental specific enhancer at the 3' end (Jacquemin et al., 1994) and a multi-component locus control region at the 5' end (Jones et al., 1995). As well, the enhancer elements within the 3' end of the human placental lactogens act as silencers of pituitary expression by the placental genes (Jiang and Eberhardt, 1997). Because mrp/plfs are closely related to the growth hormone and placental lactogen genes, it is possible that mrp/plfs are also regulated in a similar manner.
Post-transcriptional regulation of pif1 gene expression has been described in 3T3 cells and in primary mouse embryo fibroblasts (MEF) in which primary transcripts were shown to be incompletely processed or degraded in the nucleus. Stimulation of 3T3 cells by bFGF or serum resulted in an increase in mRNA levels but no change in the rate of mrp/pif transcription (Linzer and Wilder, 1987; Nilsen-Hamilton et al., 1987 Mohideen; 1995). Similarly, immortalization of MEFs resulted in increased expression of pif1 in the absence of increased rates of transcription (Edwards et al., 1987; Malyankar et al., 1996). In FGF-stimulated 3T3 cells, increased levels of pif1 mRNA could not be ascribed to a decreased rate of degradation (Mohideen; 1995). The mechanisms of post-transcriptional control of pif1 gene expression are not well understood, but appear to be at the level of nuclear stability and processing of the primary transcript (Edwards et al., 1987; Malyankar et al., 1994; Malyankar et al., 1996; Mohideen, 1995.

From the studies described here, the 5' and 3' sequences of the mrp/pif3 gene appear to be important for regulating tissue specific expression in the presence of a constitutive CMV enhancer. However, the evidence suggests that the 5' flanking sequence of the mrp3 gene also regulates splicing of the transgene in a tissue specific fashion. This conclusion is proposed because, although the hybrid intron used in these studies has been demonstrated to be efficiently processed in numerous tissues (Choi et al., 1991), the intron was only found in the entirely spliced form in the absence of the majority of the 5' flanking sequences of the mrp3 transgene. Because the extent of splicing was further
diminished in transcripts that began 5' of the normal start site, it is likely that the majority of unspliced transcripts were derived from upstream promoters.

It is possible that tissue specific transcriptional regulation and splicing of the mrp3 gene is mediated in part by competition between different promoters within the 5' flanking sequences. Competition between two promoters for activation by one enhancer has been demonstrated in transient transfections of the β- and, ε-globin promoters in the presence of the β-globin enhancer (Choi and Engel, 1988). Also, the active even skipped gene promoter acts as a boundary that prevents activation of a second even-skipped promoter in the presence of the trithorax like gene product (Ohtsuki and Levine, 1998). It is thus possible that, in the absence of tissue specific factors, ubiquitous transactivators bound to the CMV enhancer (or an as yet unidentified enhancer associated with the endogenous genes) could recruit RNA polymerase or relieve repressive chromatin structure only in relation to upstream promoters. The initiation of transcription at -142 is in agreement with the identification of a minor transcription start site identified by Linzer (Linzer and Wilder, 1987), and could be due to a TATA binding sequence identified at -170 to -162 or a TFIIId binding element at -161. Transcripts initiated at or upstream of -142 would contain at least two translational start codons (ATG) that are followed by nonsense codons prior to the first intron. These are conditions that cause nonsense mediated nuclear RNA degradation (Hentze and Kulozik, 1999; Lim et al., 1992; Maquat,
Thus the relative lack of spliced transgene transcripts derived from upstream promoters may be due to recognition by a RNA surveillance mechanism (Hentze and Kulozik, 1999) that identifies and degrades transcripts that contain nonsense codons followed by introns (Maquat, 1995; Zhang et al., 1998a; Zhang et al., 1998b). The presence of nonsense codons upstream of an intron has also been demonstrated to prevent splicing (Aoufouchi et al., 1996; Naeger et al., 1992).

It is also possible that RNA PolII is not phosphorylated on the carboxy terminal tail when it initiates transcription from these alternate upstream promoters so splicing is inefficient (Misteli and Spector, 1999; Steinmetz, 1997).

The low ratio of spliced to unspliced hybrid intron from the mrp3 promoter reporter constructs appears to contrast with results obtained with endogenous mrp/plf transcripts. The sequence differences between the hybrid intron and the endogenous introns could cause the primary transcripts to be processed differently. As well, the difference in the number of introns (4 in endogenous mrp/plf and 1 in the transgene) could effect processing. It is possible that in upstream nonsense transcripts, intron I of the endogenous mrp/plfs is also not being spliced, similar to the hybrid intron, but introns 2 through 4 in the endogenous genes mediate the decay of these transcripts. This might explain why only spliced RNAs demonstrated the upstream initiation site using RACE.

In conclusion, we have demonstrated that mrp3 flanking sequences may
direct tissue specific expression, but only under the additional influence of a heterologous enhancer. Furthermore, we demonstrate that the 5' flanking sequences of the mrp3 gene affect the number of spliced transgene transcripts in a tissue specific fashion, likely by selection of alternate upstream start sites. Because as much as 2/3 of all exonic RNA remains or is degraded in the nucleus (Jackson et al., 2000), the results demonstrated here present a provocative model of how transcriptional start site regulation could be a dominant but cryptic (due to nuclear degradation or inefficient processing) mechanism of gene regulation in mammalian cells.

References


Figures

Figure legends

Figure 1. The CMV/-1450mrp3/sis/lacZ/3.6 transgene is specifically expressed in the trophoblastic giant cells. E11 or e13 embryo and placentae were dissected from the uterus, fixed, and stained overnight for β-galactosidase activity. E11 β-galactosidase activity is demonstrated in the trophoblastic giant cell layer (TG), and is absent from the embryo, or other layers of the placenta (D=decidual; SG=spongiotrophoblast).

Figure 2. Removal of 5' and 3' flanking sequences causes aberrant expression of CMV/mrp3/constructs. Wounds were produced in CMV/-1450mrp3/sis/lac/3.6(a) or CMV/-49mrp3/sis/lac/0.6(b) transgenic mice by snipping approximately 2mm from the tip of the tail. Mice were sacrificed 3 days later and the wounds were stained for β-galactosidase activity.

Figure 3. Limited splicing of transgenic hybrid intron. 1/2 of 3 transgenic placentae from either -1450mrp3/sis/lac/3.6(lane1) or from -1450mrp3/sis/lac/3600 co-injected with a B-actin promoter/3' end cassette(lane2) or a CMV/-1450/sis/lac/600 construct(lane 3) were used for RT-PCR using primers going upstream from the lacZ gene(ULAC) and downstream from the 5'
untranslated region of the mrp3 transgene(d4263). Little spliced product(S) was observed relative to unspliced(U). Lanes 3, 4, and 5, are from the same RNA samples in the absence of reverse transcriptase.

**Figure 4. Tissue and cell specific splicing of the transgenic hybrid intron**

RT-PCR using primers which amplify between the mrp3 5' untranslated region(d4263) and the lacZ gene(ULAC), was performed on the skin(lane1) and the heart of a day13 mouse pup(lane 2), as well as isolated keratinocytes stimulated or not stimulated with KGF(lanes 3 and 4), wounded or non-wounded mouse skin(lane 5 and 6), or non-wounded skin from a line of transgenic mice containing the CMV/-49mrp3/sis/lac/.6 construct(lane 7). Lanes 8-14 are the same samples as lanes 3-7 but in the absence of reverse transcriptase. After PCR, the products were run on a 2% agarose gel and photographed under UV illumination. U represents unspliced PCR product and S represents spliced PCR product. The intermediate band between spliced and unspliced products is a hybrid of DNA strands from spliced or unspliced cDNAs which hybridized during PCR. Shown above the lanes is the ratio of spliced to unspliced product as measured by scanning densitometry of the film.
Figure 5. Alternate upstream mrp/plf transcripts are present and are differently post-transcriptionally regulated. (a) Rapid amplification of cDNA Ends (RACE) was performed on e11 embryo, total e11 placental RNA, or 17hr. FGF stimulated or unstimulated 3t3 cell nuclear RNA, using primers complementary to intron I or exon III sequences for reverse transcription, followed by two rounds of amplification using a primer complementary to exon 1 and primers specific for the C-tail. The larger sized band observed in the absence of FGF stimulation was sequenced and corresponded to a transcription start site 142 base pairs 5' of the placental start site. (b) Transcripts beginning from upstream start sites of endogenous mrp/plf genes are identified in 3t3 nuclei with and without FGF, in total placental RNA, and in total RNA from cos cells transfected with a full length mrp3 gene containing all four introns and under control of -1450 mrp3 and CMV. (c) RNAs derived from upstream start sites appear to be more prevalent in the cytoplasm than the nucleus of 3t3 cells. Lanes 1-4 3T3 are nuclear RNA from FGF stimulated(1, 2), or unstimulated(3, 4). Lanes 5-8 are cytoplasmic RNA from FGF stimulated(5, 6), or unstimulated(7, 8). Lanes 9 and 10 are from placental RNA. Odd lanes are using primers which amplify from +42 to exon 3. Even lanes use primers which amplify from -82 to exon 3.
Figure 6. 5' mrp3 sequences decrease expression and cause lower relative levels of splicing. Constructs containing either -49, -130, -230, or -1450 of mrp3 sequence downstream of the hCMV enhancer were transfected into cos-1 cells, and RT-PCR was performed as described using primers recognizing the 5' end of the hybrid intron, or either the cat or lacZ gene. SL or UL represents spliced or unspliced hybrid intron from lacZ constructs respectively. SC or UC represent spliced or unspliced hybrid intron from Svsiscat constructs.

Figure 7. Lower ratios of spliced:unspliced transcripts produced from upstream promoters.

RT-PCR was performed on RNAs from figure 6 using primers that recognize transcripts from different regions of the 5' flanking sequence of mrp3 and a lacZ specific primer.
Table 1. Transgene expression from mrp3/sis/lacZ constructs is not detectable in transgenic embryos or placenta in the absence of a strong enhancer. Transgenic embryos were identified by PCR on a portion of the yolk sac.

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<th>Construct</th>
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<th>ectopic</th>
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<th>G.C. spec. stain</th>
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Figure 1
Figure 2
Figure 3
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Figure 4
**Figure 5**

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<td>3'</td>
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**Figure 5B**

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<th>+42/lacZ</th>
<th>+42/lacZ (No RT)</th>
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<td></td>
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</table>

| spliced | 0.04    | 0.06    | 0.06     | 0.6             |
| unspliced | 2.22  | NA      | 0.06     | 0.09            |

Figure 7
CHAPTER 6: CONCLUSION

The aim of the research presented here was to gain a better understanding of the spatial and temporal expression pattern of each of the mrp/plf gene family members in vivo. Research was also performed with the goal of understanding the mechanism by which mrp3 is expressed in a tissue specific fashion. In pursuit of these goals, as detailed in the previous three chapters, a number of findings have been made that will contribute to the understanding of mrp/plf regulation in the mouse. The significance of these results as they relate to the specific aims of this project and current understanding in the field will be addressed below, as well as recommendations for studies following this work.

Tissue and temporal specific expression of different mrp/plf genes

Results presented in this thesis demonstrate for the first time that expression of three mrp/plfs (mrp3, plf1, and a newly identified mrp/plf ;mrp4) is differently regulated in tissue specific patterns in the adult mouse, and also temporally in the placenta. Mrp3 is shown to be the most abundant mrp/plf in the placenta and is also the predominant form expressed by keratinocytes during cutaneous wound repair. Plf1 is expressed at higher relative levels during early gestation and is also the predominant mrp/plf expressed in the stomach and small intestine. Mrp4 is a newly identified mrp/plf that is expressed at higher levels slightly later in gestation, and that is also the predominant mrp/plf
expressed in the ear and tail of adult mice.

**Extra-placental expression of mrp/plf genes**

What is the significance of multiple mrp/plfs expressed in particular tissue and temporal specific patterns? The identification of mrp/plfs in non-placental tissues suggests that these genes may have functions outside of pregnancy. Previously identified as a uterine growth factor, it is possible that mrp/plfs have a similar role in extra-placental tissues. Cell proliferation occurs during wound healing, the hair follicle cycle, and during the constant replacement of the lining of the gastro-intestinal tract. MRP/PLFs could play a role in the proliferation of cells in these locations, similar to one of the roles it plays during gestation. Another function could include angiogenesis, which is also a described function of placental MRP/PLFs, and which would likely be necessary in order for growing or regenerating tissues to acquire a blood supply.

Angiogenesis is a vital component of wound healing (Klein et al., 1999), and is also increased around the growing hair follicle (Mecklenburg et al., 2000), both sites where MRP/PLFs were identified. It is also possible that MRP/PLFs could act as local competitors for interaction with the IGF II/Mannose 6-Phosphate receptor. It has been demonstrated that activation of the latent form of TGFβ occurs through a mannose 6-phosphate dependent binding to the mannose-6-phosphate receptor and cleavage by urokinase (Ghahary et al., 2000; Godar et al., 1999). MRP/PLF-expressing cells could thereby locally prevent the effects of
TGFβ—by competing for binding to the mannose-6 phosphate receptor to prevent TGFβ-activation. MRP/PLF could also act intracellularly to inhibit differentiation of cells that are needed to proliferate, as in mouse myoblasts. (Muscat et al., 1991; Wilder and Linzer, 1989) MRP4 is distinct from the other mrp/plfs in that it is less glycosylated and is missing a small helical region in the C terminus that is found on the other MRP/PLFs. Therefore, it is possible that MRP4 may have a different function than MRP3 or PLF1. It will be important in this regard to find out if MRP4 contains mannose-6-phosphate (M-6-P) residues and binds to the M-6-P receptor and/or interacts with the mrp receptor identified in uterine cells (Nelson et al., 1995). The identification of predominantly mrp4 expression in hair follicles of tails and the ears of mice, two relatively hairless areas, and the expression of mostly mrp3 in the hair follicles of the backskin or during wound healing also encourages speculation that these proteins may have different functions specifically in the skin and possibly with relation to hair growth.

Growth hormone and prolactin are expressed in tissues other than the pituitary, however, there have been few reports demonstrating extra-placental expression of the placental members of the PRL/GH family. Because MRP/PLFs have not been demonstrated to bind the prolactin or growth hormone receptors, it may not be useful to suggest functions of extra-placental MRP/PLF expression based upon functions of extrapituitary prolactin expression. Suggestions as to the functions of MRP/PLF in extra-placental tissue will likely come from more
thorough analysis of MRP/PLF receptor localization. Immunohistochemistry has demonstrated MRP/PLF protein in the tissues expressing mrp/plf mRNAs, and it is likely, but not definite, that the cells staining positively for MRP/PLF protein are expressing MRP/PLF proteins. To better determine which cells are producing specific mrp/plfs and which cells are binding it—the protein will require in situ hybridization or in situ RT-PCR, combined with analysis of MRP/PLF binding sites in or near these tissues. In situ RT-PCR would be especially useful, because specific mrp/plf forms could be identified by choosing primers that only recognize one form, whereas in situ hybridization would not distinguish between different mrp/plf types. Identification of MRP/PLF binding sites, and determination of whether M-6-P is involved in binding will be very important for the identification of a function for extra-placental MRP/PLF.

Functional knockouts of mrp/plf expression may also be revealing. It would be difficult however, to knockout all mrp/plf expression using homologous recombination, because there are at least four highly homologous genes. However, because each mrp/plf is expressed with a different tissue distribution, it may not be necessary to knockout all of the mrp/plf genes. It may be more revealing to knockout individual mrp/plf genes and determine how each contributes to the physiology of the mouse. Another option, made possible by the high homology between mrp/plfs, is that all the mrp/plf mRNAs may be targeted at once for antisense or ribozyme mediated destruction. As the technology for
producing effective ribozymes continues to improve, it may be possible to effectively destroy mrp/plf messages in specific tissues or at specific times using tissue specific or inducible promoters to express a ribozyme which recognizes all the forms of mrp/plf. In fact, numerous promoters have been characterized that express transgenes in specific tissues, including the outer root sheath of hair follicles (Pena et al., 1999; Soler et al., 1996), keratinocytes at the edge of healing skin wounds (Mohan et al., 1998; Munaut et al., 1999), tail skin (Werner et al., 1993), and trophoblastic giant cells (Shi et al., 1997). Because of its very high expression in the placenta, to reduce mrp/plf levels in the placenta using antisense or ribozymes would likely require a very strong promoter.

In exploring the function(s) of MRP/PLFs, it also might be informative to over express a particular MRP/PLF in a specific tissue and determine if there is a phenotype. For instance, mrp4 could be over-expressed in the backskin of mice, or mrp3 could be directed to the tail and ears. These studies could help to determine if the natural pattern of segregation of these forms serves a purpose or is just a result of coincidental accumulation of transcription factors that activate a specific form of mrp/plf expression.

**MRP/PLF expression in the placenta**

The functions suggested for MRP/PLF in the placenta and the uterus include angiogenesis and stimulation of uterine growth respectively (Jackson et al., 1994; Nelson et al., 1995). How does this relate to the temporal expression
patterns of different mrp/plfs in the placenta during gestation? The most notable characteristic of differential mrp/plf expression that may relate to function, is the day 12 peak of mrp4 expression. This is likely the source of a lower MW mrp identified by Western blot that is found at the highest levels on day 13 (Fang et al., 1999). If MRP4 has a similar function to the other MRP/PLF forms, this expression pattern could contrast with the current model proposed by Linzer, in which MRP/PLF angiogenic activity decreases at the same time that expression of a angiogenesis inhibitor called Proliferin Related Protein (PRP), increases in order to shut down neovascularization and restrict invasiveness of the placenta (Jackson et al., 1994; Linzer and Fisher, 1999). Interestingly, PRP (Colosi et al., 1988) and Mrp4 are expressed in the placenta at the same time.

Unlike the more highly glycosylated forms of MRP/PLFs, MRP4 is not found in the amniotic fluid. This observation suggests that MRP4 is either rapidly degraded, bound to a receptor, or is not effectively transported to the fetus. However, the precise location of mrp4 expression within the placenta and how it relates spatially to PRP secreting cells is not clear. So it is still possible that MRP4 and PRP have very local but opposite effects. It is also possible that the lack of M-6-P residues prevents MRP4 angiogenic activity or even causes MRP4 to act similarly to PRP, as an angiogenesis inhibitor.

Identifying a function for different temporal expression patterns for MRP4 and the other MRP/PLF forms will depend upon the demonstration of binding
activity for MRP4, stimulation of receptors by the different MRP/PLF proteins, as well as the localization of these receptors in specific tissues and cell types. It would also be of interest to perform in situ RT-PCR with *mrp4*-specific primers in order to determine if this gene is expressed in the same cells as the other *mrp/plfs*. Specific expression of *mrp/plf* RNA in the giant cells have been demonstrated for day 10 placentae by *in situ* hybridization (Lee et al., 1988), but analysis of later stage placentae, when *mrp4* is produced have not been published.

Other placental GH/PRL family members, including the mouse placental lactogens I and II, and at least 10 prolactin like proteins (PLPs) (Dai et al., 1996; Deb et al., 1991; Deb and Soares, 1990; Deb et al., 1989; Lin et al., 1997a; Lin et al., 1997b; Muller et al., 1998a; Muller et al., 1998b) are also expressed in particular tissue specific and temporal patterns during gestation (Soares et al., 1998). While differences have been noted in the ability of mPLs I and II to effect gene expression in the mammary gland and corpus luteum through interaction with PRL receptors, much less is known about the reason for differential *plp* gene regulation. One *plp* family member has been shown to bind to natural killer cells and to prevent cytolytic activities of natural killer cells within the decidua (Muller et al., 1999). Thus, the *mrp/plfs* and the *plps* represent at least 13 different non-classical GH/PRL related genes in the mouse placenta which are regulated in different cell and temporal specific patterns. Determining the
functions of these proteins, and how spatial and temporal regulation of these genes relates to function will be important for gaining a better understanding of mammalian reproduction.

Potential mechanisms of tissue specific variation in mrp/plf expression

The mechanisms by which mrp/plf genes express in particular cells or tissues are also of interest. Much work has been done in cultured cells using different mrp/plf promoters to understand gene expression in response to mitogens or serum. Prior to this work it was not completely understood which mrp/plfs were expressed in the placenta, nor which promoters produced the mRNAs expressed in the placenta or in 3t3-3T3 cells. Therefore, analysis of mrp/plf promoter activity in cultured cells, while valuable in contributing to understanding transcriptional regulation in response to mitogens, could not be compared to activity of the endogenous promoters to gain a understanding of these genes in a physiological context. In this work, the promoters for each expressed mrp/plf gene have been identified as well as sites of tissue specific expression. The promoter known as plf42, which is activated by serum in a transfected liver cell line (Linzer and Mordacq, 1987), is the promoter for plf1. The promoter known as plf149, which is not stimulated by serum in transfected liver cells (Linzer and Mordacq, 1987), is the promoter for mrp4. The promoter for mrp3 was already identified as being contiguous with the remainder of the mrp3 gene (Connor et al., 1989).
The upstream region of \textit{mrp3} contains an FGF response element that allows it to be specifically activated in response to FGF while the other two promoters are not activated (Mohideen et al., 1999). Despite 98% homology in the sequenced regions of these promoters, each promoter is regulated differently from the other in response to particular stimuli. These differences in activity have been shown to be the result of slight alterations in nucleotide sequence that result in different cis-acting elements. While the physiological significance of these differences of transfected promoter activity in response to treatment are unknown, the demonstration of tissue specific and temporal regulation of these genes will allow analysis of these differences in comparison to the physiologic context in which these genes are expressed.

Although caution should be exercised when comparing the regulation of transfected promoters to the regulation of endogenous genes, some of the results obtained regarding expression patterns of endogenous \textit{mrp/plf} genes encourage speculation. For instance, the specific FGF response element found in the \textit{mrp3} gene might be correlated with the specific expression of \textit{mrp3} in healing skin wounds. In healing wounds or in the cycling hair follicle, expression and functions of members of the FGF family including bFGF (Ortega et al., 1998), FGF5 (Suzuki et al., 2000), and particularly KGF (Werner et al., 1992; Werner et al., 1994) have been demonstrated. Furthermore, in chapter four it was demonstrated that KGF stimulates \textit{mrp/plf} synthesis in primary cultures of
keratinocytes, suggesting that members of the FGF family might stimulate
mrp/plf synthesis in healing wounds. It is possible that this stimulation occurs in
part through the specific FGF response element previously identified in
transfected 3T3 cells.

However, there is clearly more to regulation of the endogenous mrp/plf
genes than in the first 350 basepairs of upstream sequence. This is illustrated
by the demonstration that 1510 basepairs of mrp3 upstream sequence fails to
direct detectable reporter gene expression to the placenta in transgenic embryos
(chap 5) and that 3T3-3T3 cells produce predominantly plf1 in response to bFGF
stimulation (chap 2), even though the mrp3 promoter is stimulated by bFGF in
transfected cells. Further complicating the issue is that a post-transcriptional
nuclear mechanism has been determined for the FGF induced increase in plf1
expression (Mohideen, 1995), suggesting that the plf1 promoter is constitutively
active in these cells. Mrp3 is also expressed at low levels in 3T3 cells, but it is
not known whether endogenous mrp3 is regulated transcriptionally or post-
transcriptionally, due to the relatively low abundance of mrp3 mRNA in
comparison to plf1 in 3T3 cells.

What and where are the differences between these genes that allow mrp3
to be the most abundant mrp/plf in skin wounds and the placenta, mrp4 to be the
most abundant in the ear and tail, and plf1 to be most abundant in 3T3 cells and
the alimentary tract. This problem may be approached from different strategies.
One could ask, for instance, if particular transcription factors affect individual \( mrp/plf \) genes differently. Proteins that have been shown to play a dramatic role in \( mrp/plf \) expression in the placenta are GATA factors, for which there are binding sites in the \( mrp/plf \) promoters. While direct activation of the \( mrp/plf \) promoters has not been demonstrated, functional knockouts of GATA factors, especially GATA 2, decrease \( mrp/plf \) expression up to 80% (Ma et al., 1997). It is likely that GATA factors effect \( mrp3 \) expression, because this is the predominant form in the placenta, and such a dramatic decrease would not be seen if \( mrp3 \) were not affected. It is not known however, if expression of each \( mrp/plf \) is effected equally by GATA factors. Nor is it clear if GATA factors directly interact with the \( mrp/plf \) promoters or if they activate expression of some other gene that stimulates \( mrp/plf \) expression. It would be fairly simple to use the RT-PCR restriction digest assay described in this work to determine if GATA factors specifically regulated a particular \( mrp/plf \), or affected all \( mrp/plfs \) equally. It would also be possible to transfecGATA factors or other placental transcription factors into 3T3 cells to determine if different \( mrp/plfs \) could be induced, or measure the activity of the different \( mrp/plf \) promoter constructs in response to co-transfected transcription factors. These kinds of experiments could identify proteins that are involved in the differential expression of the \( mrp/plf \) genes. Other transcription factor knockouts have also been created that could-affect \( mrp/plf \) expression including cJun, cEBP, and c-ETs-2 for which there are binding motifs in the
mrp/plf promoters. The relative and total levels of each mrp/plf in knockout mice could provide valuable information on how these genes are regulated tissue specifically.

The second way to identify mechanisms involved in differential mrp/plf expression is to compare the effects of the sequences surrounding each mrp/plf gene on cell type specific expression. In cell culture, upstream sequences of up to -650 b.p. have been used to identify elements involved in response to various growth factor or hormone treatments (Mohideen et al., 1999). In this case, sequences involved in differential regulation by growth factors were easily identified because the mrp/plf genes are highly identical. These genes could thus become very useful tools in studying transcriptional regulation, because each gene already contains mutations that differentially effect growth factor or cell type specific expression.

To determine if the sequences surrounding the mrp/plf genes contain elements involved in cell specific expression, the overall levels of expression, relative to a cotransfected reporter, should be measured for each promoter in each cell type to be tested. This analysis could be done in stably transfected keratinocyte cell lines, trophoblast cell lines, or 3T3 cells. This kind of analysis could possibly identify promoter elements involved in cell specific expression of mrp/plf types. However, the best way to analyze the mrp/plf genes for elements contributing to cell or tissue specific expression in the context of which they are
expressed would be to analyze the activity of different mrp/plf promoters in transgenic mice. This analysis was initiated using the mrp3 promoter in the research presented here.

**Analysis of the mrp3 promoter in transgenic mice**

As demonstrated in chapter 5, 1514 basepairs of mrp3 promoter along with 3.6 kb of 3' sequence is not sufficient to express detectable reporter gene expression in the placentae of transgenic embryos. These results are further evidence for the contribution of as yet unidentified sequences to endogenous mrp/plf expression. Addition of the CMV enhancer to the 5' end of the gene however, did allow tissue specific expression of the mrp3 promoter when 3 kb of mrp3 downstream sequence was present at the 3' end of the lacZ gene. These results suggest two important aspects of mrp3 regulation. First, the mrp3 promoter requires an enhancer to be expressed in vivo. The enhancer element is evidently not present in the sequences immediately surrounding the coding sequences of the mrp3 gene. This requirement is somewhat similar to the human growth hormone gene cluster, which is regulated by a locus control region and a series of DNAse hypersensitive sites located 15 to 30 kb away from the most 5' gene. (Jones et al., 1995). Second, a 1514 basepair mrp3 sequence including the mrp3 promoter, and the 3' 3.6 kb non-coding region of the mrp3 gene contain regulatory elements capable of directing tissue specific expression of the transgene, but only in the presence of an additional enhancer. It is particularly
interesting that, although the CMV enhancer is active throughout the developing fetus (Baskar et al., 1996), the mrp3 sequences are able to not only direct giant cell expression, but to prevent expression in non-giant cells. Thus, to better understand cell specific expression of mrp/plfs, it will be important to identify the endogenous enhancers for the mrp/plfs, as well as determine how the more proximal sequences are able to direct tissue specificity in the presence of an enhancer.

**Finding endogenous mrp/plf enhancers**

As a first step towards analyzing further sequences of the mrp/plf genes for enhancers, cosmids containing each mrp/plf gene have been identified. The identification of cosmids containing individual mrp/plfs will be very useful for investigating the differential regulation of these genes. For instance, it will now be possible to stably transfect a non-mouse trophoblast cell line (such as RCHO cells) with individual cosmids, and correlate expression of mrp/plfs with DNAse hypersensitive sites specifically induced upon giant cell differentiation. This could provide evidence of a giant cell specific enhancer. Whereas identification of DNAse hypersensitive sites in mouse cells containing the cluster of highly homologous mrp/plf genes would be rather complicated due to the high homology and multitude of shared restriction sites for each mrp/plf, the analysis of one 40 kb fragment at a time containing a known mrp/plf would more easily define the location of a DNAse hypersensitive site. It will also be possible to co-
transfect cells with equal amounts of different mrp/plf cosmids and determine, using the diagnostic RT-PCR assay, if there are specific elements within those cosmids that enable a particular gene to be more active. Larger upstream regions containing potential regulatory elements could then be subcloned and tested in transgenic mice for tissue specific expression.

**Tissue specific expression with a ubiquitous enhancer**

While the results presented in chapter 5 suggest that an enhancer is required for mrp3-promoter activity in vivo, these results also show that the 1514 b.p. of upstream and 3.6 kb downstream mrp3 sequence are able to direct tissue specific expression. How do the mrp3 sequences direct tissue specific expression in the presence of the CMV enhancer? In most models of transcriptional regulation by enhancers, transcription factors bound to the enhancer interact with trans-acting factors associated with the promoter to increase transcriptional initiation by RNA polymerase II. Some models suggest that the main function of enhancers is to overcome inhibitory chromatin structure at weak promoters (Majumder and DePamphilis, 1995), and allow an open chromatin structure in which RNA pol II may more easily bind. Other models suggest that enhancers may recruit transcription factors and coactivators that then loop to contact and activate the RNA pol II machinery at the promoter (Blackwood and Kadonaga, 1998). Both of these models may occur to some degree. If these models are applied to the CMV/mrp3 transgene expression
during midgestation, the results would suggest that the CMV enhancer is best able to functionally interact with the mrp3 promoter in giant cells, and that the likelihood of this interaction occurring is effected by the mrp3 promoter sequence included in the transgene, as well as surrounding chromatin structure at the transgene insertion site. Evidence is provided here which suggests that elements within the surrounding mrp3 sequences can prevent CMV induced activity of the promoter in non-giant cells. When the 3' 3kb fragment is removed from the mrp3 transgene construct, expression becomes more aberrant, and in some cases giant cell expression is lost. This result suggests that some tissue specific regulation is associated with the 3' 3kb fragment. Furthermore, in transfected COS cells, and also in transgenic embryos, as upstream sequences are removed from the 5' end of the mrp3 gene, expression levels and non-specific expression of the transgene increases respectively. These results suggest that 5' sequences may also inhibit functional interaction with the mrp3 promoter.

How do these 5' or 3' sequences inhibit the CMV enhancer from interacting with the mrp3 promoter? In the hGH family placental members, pituitary expression is repressed by specific elements in the 5' ends of the genes, as well as the 3' end (Nachtigal et al., 1993). Curiously, the same repressor in the 3' end acts as an enhancer of placental expression (Jiang and Eberhardt, 1997). Another possible reason that the mrp3 5' sequences inhibit
CMV promoter activity could be that the probability of interaction with the enhancer is decreased simply because of the increased distance between the mrp3 promoter and the enhancer. For this reason, it will be important to mutate or replace elements of the mrp3 gene that are suspected of being involved in tissue specific expression, rather than remove them. These elements could include GATA factor binding elements, the AP-1 sites, AP-2 site, or the FGF response element, all which are involved in either giant cell gene expression (Ma et al., 1997; Schorpp-Kistner et al., 1999; Shi et al., 1997) or have been demonstrated to effect mrp/plf expression (Mohideen et al., 1999).

**Mrp3 upstream sequences effect splicing of the transgene**

Another explanation as to how mrp3 sequences may play a role in promoter activity in the presence of the CMV enhancer is suggested by analysis of the RNA produced by different deletion constructs. In transfected cells, it was demonstrated that the presence of the upstream region of the mrp3 gene represses expression of reporter gene activity; and decreases splicing of a downstream heterologous intron. It was also demonstrated that RNA transcripts originating further upstream than the most 3' transcriptional start site contained a lower ratio of spliced to unspliced transcripts than of transcripts that began after the normal start site. This suggests that there are upstream promoters that can produce non-functional transcripts. It is thus possible that upstream promoters compete for interaction with the CMV enhancer. It would then follow, that, in the
tissues in which expression of the transgene is demonstrated, a promoter which results in functional transcripts is being chosen over other possible promoters. Thus, in the absence of tissue specific transcription factors, mrp3 sequences could prevent non-tissue specific expression in the presence of a ubiquitous enhancer by competing for or actively preventing enhancer activity from interaction with the correct promoter. In support of this model, it is demonstrated that the ratio of spliced to unspliced heterologous intron is increased in isolated keratinocytes when compared to whole skin, while the intron is completely spliced in the whole skin of mouse containing the transgene missing the upstream promoters of the mrp3 gene. Splicing is also higher in skin containing cycling hair follicles than in the heart of the same mouse. While further experiments must be done to demonstrate that upstream promoters are acting as functional repressors through competition and the production of nonsense transcripts, this is a model that could account for what appears to be post-transcriptional regulation of the plf1 message, but transcriptional regulation of transfected promoters. If, in 3T3 cells, there is an enhancer that is constitutively active, and is competed for by functional and decoy promoters, it is possible that stimulation with mitogens could increase expression or activation of transcription factors or accessory factors that favor activation of the correct promoter. Mitogen stimulated transcription factors could increase MRP/PLF expression through occlusion of the decoy promoter(s), by specifically derepressing the
correct promoter, and/or by actively recruiting enhancer activity or RNAPol II to the correct promoter. In the whole mouse or embryo, nuclear run-off analysis of the mrp/plf genes has not been reported. However, when mouse embryo cells are dispersed, there is no difference in transcription levels of mrp/plfs between these primary non-mrp/plf producing cultures, and 3T3 cells, which produce high levels of plf1 (Malyankar et al., 1994; Malyankar et al., 1996). Thus, it is possible that this type of regulation is occurring in intact mouse tissues as well.

Competition between promoters for enhancer activity has been suggested as a mechanism by which the genes which comprise the globin locus are regulated by the locus control region (Choi and Engel, 1988), and a similar process has recently been demonstrated in Drosophila, by which one upstream evenskipped promoter acts as a boundary element for activation of a further downstream evenskipped promoter (Ohtsuki and Levine, 1998). Furthermore, multiple alternate upstream initiation sites have been demonstrated on many genes, including the prolactin promoter.

Typically alternate promoters result in either a longer 5' untranslated region or an extra exon that may or may not be translated. But what happens to transcripts that originate from upstream promoters with translational start codons that result in nonsense containing transcripts? The results presented here suggest that these transcripts may either not be spliced or may be degraded soon after splicing, resulting in a higher ratio of unspliced to spliced message. In
most eukaryotic organisms that have been examined, transcripts containing nonsense mutations that are upstream of an intron are degraded either in the cytoplasm (yeast) or in the nucleus (mammals) (Hentze and Kulozik, 1999; Maquat, 1995). So, it is possible that spliced transcripts from upstream promoters such as mrp3 are degraded in the nucleus and that the apparent increase in relative levels of unspliced message from upstream promoters is the result of a lower stability of the spliced nonsense containing transcripts. This provides the basis for a fine tuning mechanism of transcriptional regulation (figure 1) by which transcription factors or enhancers with a high potential to induce transcription could be present on a gene and still not result in functional transcripts unless particular transcription factors direct RNA polymerase activity to the correct promoter. The implications of this could be important not only to understanding mrp/pif regulation, but eukaryotic gene regulation in general, as it would suggest a previously unrecognized mechanism of gene regulation which could largely go unnoticed, because the transcripts from decoy promoters would be degraded rapidly. In fact, it has been determined that 95% of RNA pol II RNA transcribed in the nucleus is also degraded in the nucleus. While a large portion of this degradation is accounted for by removal and degradation of introns (~80%), it has been estimated that only a third of exon containing RNA makes it out of the nucleus as well (Brandhorst, 1974; Jackson et al., 2000; Soeiro, 1968). Thus, nuclear RNA decay appears to be a very important level of gene
A. Promoter not accessible in absence of enhancer

B. Nonsense promoter usage in absence of tissue specific factors

C. Tissue specific factors mediate correct promoter usage
regulation of which relatively little is understood. For that reason, it will be important to follow up these results with experiments that will conclusively demonstrate what is happening to upstream nonsense containing transcripts in regards to stability and splicing. It will be even more important to determine if this mechanism is actually occurring on the endogenous mrp/plf genes, and to determine how many other genes this mechanism may effect.

To determine if the mrp3 upstream transcripts are degraded by the nonsense mediated decay pathway, it will be necessary to inhibit nonsense mediated decay, and examine the levels and splicing of upstream nonsense transcripts. This can be done by cotransfecting the mrp3 constructs described here with a dominant negative form of the UPF-1 gene. The wild type form of this gene encodes a human homologue of a yeast protein known to be involved in nonsense mediated RNA decay, while a mutant form, which has a cysteine to arginine mutation acts as a dominant negative and has been shown to abrogate nonsense mediated decay (Sun et al., 1998). An increase in spliced message from upstream transcripts in the presence of dominant negative UPF-1, but not wild type UPF-1, would be evidence that the nonsense mediated decay pathway is involved in removing spliced transcripts from upstream promoters. This analysis could also be performed on endogenous mrp/plf message by stably transfecting or transiently transfecting high levels of wild type or dominant negative UPF-1 into 3T3 cells, and assaying for levels of upstream transcripts.
This could even be done in transgenic mouse embryos by ubiquitously expressing high levels of dominant negative UPF-1, and assaying RNA from the embryo proper for mrp/plf levels. Clearly there are many experiments that could be designed to investigate this phenomenon, including mutation of upstream promoters, removal of translational start codons from upstream regions, and removal of introns, all which could provide evidence for the model suggested. Though more work is needed to understand how the mrp3 upstream sequences are involved in splicing, I believe that dissection of this mechanism could provide a useful contribution to the study of gene regulation.

In conclusion, it has been demonstrated that three members of the mrp/plf gene family are regulated in different tissue and temporal specific patterns. The finding that these genes are differentially expressed in adult epithelia suggests further functions than those previously proposed for placental mrp/plf. It is also now clear that while proximal regions of the mrp3 gene may be involved in tissue specific expression, an enhancer is necessary for the high level expression demonstrated for the endogenous mrp/plf genes in the placenta. Finally a model is provided which suggests how tissue specific expression in the presence of a strong ubiquitous enhancer might be achieved. This model also reconciles observed discrepancies between interpretations of studies of post transcriptional regulation of endogenous mrp/plf transcripts and studies of transcriptional control of transfected promoters. Identification of functions for different mrp/plf genes in
different tissues, and characterization of the endogenous enhancer(s) and how it interacts with the proximal mrp/plf promoters to regulate expression should now be the focus of studies following this work.

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APPENDIX

IDENTIFICATION OF PROMOTERS FOR THE MRP/PLF GENES

Materials/Methods

Cosmids containing \textit{mrp/plf} hybridizing sequences (kindly provided by Dan Linzer) as well the \textit{mrp3} gene from -1450 to + 300 basepairs past the poly A site (kindly provided by Dave Denhardt) and genomic DNA, were amplified using primers which recognize from exon 3 (+307 to +332) \texttt{acaaaaagcccatgagatgcaatac} and from exon 4 \texttt{5'actcactagatgtccagagg3'} (+453 to+433). PCR amplified fragments were then digested with EcoRI for four hours at 37°C, then digested with either BsoFI or BstXI as before. The fragments were analyzed on a 2% gel. Clones which produced pcr products which digested in a pattern consistent with a particular \textit{mrp/plf} cDNA were sequenced across the promoter region using a primer (ue1-2 \texttt{caggagcatgttgaatcg}) which recognized the 5' end of the three \textit{mrp/plf}s identified so far.

Identification of the \textit{mrp4} and \textit{plf1} promoter

To identify the \textit{mrp4} promoter a series of cosmid clones were screened for the presence of the \textit{mrp4} gene by several PCR based assays. First, diagnostic PCR was performed between exons 3 and 4 on isolated cosmid clones containing \textit{mrp/plf} hybridizing genes in order to identify specific \textit{mrp/plf} containing cosmids. As shown in figure 1, individual clones were identified which
produced the predicted restriction digest pattern for each of the mrp/plfs. Of six cosmids which contained a full length mrp/plf gene, two gave patterns specific for plf1, one digested as plf2, two gave mrp3 specific patterns, and one digested as would be expected for mrp4. Clones which produced PCR products that gave mrp/plf specific digestion patterns were used as templates for PCR and sequencing across the promoter region. By this method, the promoter known as plf42 was shown to correspond to plf1, the promoter plf149 corresponded to mrp4, and the mrp3 promoter corresponded to mrp3 cDNA. The cosmid containing the plf2 gene appeared to contain a promoter with the same sequence as mrp3, however, because a cell type or tissue which expressed exclusively plf2 was not available to confirm the linkage, it is possible that the promoter sequence identified in the cosmid as mrp3 is not contiguous with the plf2 gene. To confirm that the promoter regions were actually contiguous with the genes encoding the different mRNAs, RT-PCR was performed using a 5' primer which would amplify transcripts initiating at upstream promoters located between 140 or 230 basepairs of the normal transcriptional start site(not shown). When performed on the tail, the promoter for mrp4 was confirmed to be plf149. Similarly, 3t3 cells, which produce predominantly plf1, produced upstream transcripts which corresponded to the plf42 promoter, and the placental mrp3 cDNA, was linked to the mrp3 promoter, as expected.
Figure 1