The Creation and Analysis of Protein Tyrosine Phosphatase Epsilon Mutant Mice

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Abstract

Addition and removal of phosphate from tyrosyl residues of proteins is an important mechanism for regulating the function and localization of proteins within the cell. Protein tyrosine phosphatases (PTP) are the family of enzymes that function to remove the phosphate specifically from phosphotyrosyl containing proteins. Protein tyrosine phosphatases act in concert with protein tyrosine kinases to provide intricate mechanisms for the regulation of cellular functions such as proliferation, differentiation, cell adhesion, and metabolism.

This thesis revolves around the genetic analysis of protein tyrosine phosphatase-epsilon. This PTP exists as two isoforms - a transmembrane-containing receptor-like PTP having a small extracellular domain linked to two tandem phosphatase domains via a transmembrane segment, and an intracellular form containing a unique N-terminus attached to the two tandem phosphatase domains.

The juxtamembrane region of the receptor-like form is common with the intracellular form and is defined as two exons in the mouse genome. Through the deletion of the transmembrane and first juxtamembrane exons of the Ptpre gene in mouse R1 embryonic stem cells using homologous recombination followed by Cre mediated excision of LoxP flanked exons we have generated mice mutant for these two forms of PTP-epsilon.

The objectives of this thesis is to determine the functional role of the isoforms of protein tyrosine phosphatase epsilon (PTP-ε) through loss-of-function analysis in the mouse, and to test the hypothesis that murine PTP-ε is
required for embryonic development and to determine if the gene product is required for reproduction. A further aim is to examine redundancy issues between the highly related protein tyrosine phosphatases, PTP-ε and RPTP-α.

\[ Ptpre^\Delta/Ptpre^\Delta \] mutant mice are viable and fertile. Gross anatomy and histological analysis of homozygous mutant mice shows no difference from wildtype.

Protein tyrosine phosphatase-epsilon shows the strongest amino acid similarity to the receptor-like PTP-alpha. The high degree of similarity between these two molecules suggest that functional redundancy may occur. In collaboration with Dr. Jan Sap (NYU) we crossed the \( Ptpra^\Delta/Ptpra^\Delta \) mutant mouse to the \( Ptpre^\Delta/Ptpre^\Delta \) mutant mouse to create double knockout mice. Preliminary studies indicate that these mutant mice are viable, fertile and exhibit no apparent difference from wildtype with respect to behavior.

Identification of subtle phenotypes in the laboratory context can be difficult, as important function of genes may not reveal themselves without the appropriate stresses or environment. This implies that a phenotype resulting from a particular gene knockout is present but may not be detectable by conventional methods.
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Abbreviations

AP   alkaline phosphatase
BCR  B cell receptor
BHK  baby hamster kidney
bp   base pair
CAH  carbonic anhydrase homology
CK   creatine kinase
ESC  embryonic stem cell
FACS fluorescence activated cell sorting
FGF  fibroblast growth factor
FNIII fibronectin type III
H&E  haemotoxylin and eosine
Ig   immunoglobulin
IR   insulin receptor
IRES intra-ribosomal entry site
LIF  leukemia inhibitory factor
MAM region of homology to meprin, A5 glycoprotein and RPTP-μ
MKP  MAP kinase phosphatase
NGF  nerve growth factor
PCR  polymerase chain reaction
PBS  phosphate buffered saline
PTP  protein tyrosine phosphatase
RT   reverse transcription
RTKs receptor tyrosine kinases
PTK  protein tyrosine kinases
SDS  sodium dodecyl sulphate
SH2  Src homology domain 2
SH3  Src homology domain3
SOS  Son of Sevenless
TCR  T cell receptor
WT   wildtype
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Chapter 1: Introduction

1.1 General Overview

Reversible phosphorylation of tyrosine residues of proteins within the cell is central to regulation of proliferation and differentiation as well as numerous other cellular phenomena. This regulated phosphorylation permits the allosteric modification of protein function and allows the transient interaction of numerous molecules to facilitate linkage of signal transduction pathways, cytoskeletal reorganization and coordinated regulation of gene expression.

1.2: Phosphorylation in Signal Transduction

A wide variety of complex signal transduction events occur within cells as result of ligand-mediated receptor stimulation. Which events are activated is
dependent on the receptor involved, the cell type involved as well as its state of
differentiation and its in vivo context, and can be impacted in either a positive or
negative way by other signaling events. Addition and removal of phosphate
from proteins within a cell is an important mechanism for regulating the function
and localization of proteins within the cell (Sun and Tonks, 1994). A large
variety of enzymes has evolved which are able to catalyze either the addition or
the removal of phosphate moieties from proteins. Within the cell, phosphate can
exist covalently bound to histidine, serine, threonine or tyrosine residues on
proteins with the majority being bound to serine and threonine (Roach, 1991).
Less than 0.05% of the total protein phosphorylation is found on tyrosine
residues in unstimulated cells (Hunter and Sefton, 1980). The level of
phosphotyrosine can increase up to 40 fold after factor stimulation or cell
transformation (Ushiro and Cohen, 1980). Protein phosphorylation is often
transitory and its regulation provides one of a number of mechanisms through
which cellular events can be controlled. Protein tyrosine phosphatases act in
concert with protein tyrosine kinases to provide intricate mechanisms for the
regulation of cellular functions such as proliferation, activation and
differentiation (Figure 1.1).
1.2.1: The Protein Tyrosine Kinase Super family

A great deal of information concerning the structure and function of protein kinases has been established over the last two decades. This body of work points to protein kinases as being essential to the transmission of signaling information from the surface of the cell to the nucleus by a large number of distinct and sometimes overlapping pathways. Based on their substrate preferences protein kinases are divided into those that phosphorylate serine and threonine residues (the serine/threonine kinases), those that phosphorylate tyrosine residues, and the group of so called dual specificity kinases that catalyze the addition of phosphate onto all three amino acid residues.
Many protein tyrosine kinases (PTKs) exist as either receptor PTKs or intracellular PTKs. Activation of receptor tyrosine kinases (RTKs) by ligand binding leads to the tyrosine phosphorylation of a variety of proteins substrates involved in the various signal transduction pathways. In these molecules the kinase domain is an intrinsic feature of the receptor itself. Examples of growth factor receptors include epidermal growth factor receptor (EGFR), insulin receptor and platelet-derived growth factor receptor (PDGFR). Ligand binding to the extracellular domain of RTKs leads to dimerization of the receptor and activation of the kinase domain via an autophosphorylation mechanism (Ullrich and Schlessinger, 1990). Auto-phosphorylation occurs through an intermolecular mechanism in which the kinase domain of one receptor chain cross-phosphorylates the intracellular portion of the other receptor chain. Cytosolic domain phosphorylation sites may be found both within and outside of the kinase domain. Phosphotyrosine residues either act as sequence-specific docking sites for various cytoplasmic signaling proteins or serve to regulate the catalytic activity of the kinase domain. Docking of signaling molecules is mediated through distinct protein domains. Src homology-2 (SH2) domains and phosphotyrosine binding (PTB) domains interact in a specific fashion with phosphotyrosine residues within receptor kinases and other molecules (Pawson, 1994).

Cell surface receptors exist that do not contain intrinsic tyrosine kinase activity like the growth factor receptors but have associating cytoplasmic tyrosine kinases that mediate phosphorylation of substrates upon ligand binding. The T cell and B cell antigen receptors and cytokine receptors orchestrate signal transmission by attracting cytoplasmic tyrosine kinases to mediate substrate phosphorylation. The multi-component cytokine receptor family mediate
tyrosine phosphorylation signaling events through the receptor associated Janus kinases (Jaks). This family includes Jak1, Jak2, Jak3 and Tyk2. Jaks physically associate with the intracellular portion of the cytokine receptors.

1.3: The Protein Tyrosine Phosphatases Superfamily

Protein tyrosine phosphatases are the family of enzymes that catalyze the removal of phosphate from phosphotyrosyl-containing proteins. Protein tyrosine phosphatases can be broadly divided into three groups. These are the dual specificity PTPs, the low molecular weight PTPs, and the phospho-tyrosine specific PTPs (Zhang and Dixon, 1994; Fauman and Saper, 1996; Jia, 1997). The members of these three groups, in spite of their very limited overall sequence similarity, share a minimal common active site motif comprised of a cysteine followed by five residues of any amino acid type and then an arginine (designated as CXXXXXR or CX5R). The different families of protein tyrosine phosphatases can be divided based on the extended sequence homology of the minimal active site motif (Figure 1.2).
Figure 1.2: The protein tyrosine phosphatase superfamily, defined by the presence of minimal essential active site motif, can be divided into three subfamilies.
1.3.1: Dual Specificity Phosphatase Family

This group is composed of enzymes that act on phosphotyrosyl residues as well as phosphoserine/threonine residues. The dual-specificity protein tyrosine phosphatases are structurally and evolutionarily distinct from the serine/threonine phosphatase family. They can be further sub-divided into the highly related cdc25 family of cell division control gene products (Draetta and Eckstein, 1997), and the structurally distinct VH1-like (Vaccinia virus late H1 gene product) family of dual specificity phosphatases (Guan and Dixon, 1993).

The extended active site motif for dual-specificity protein tyrosine phosphatases is represented by the consensus VXVHCXXGXSRSXTXXX AYLM. Members of the dual specificity phosphatases are listed in Table 1.1.

The cdc25 dual specificity protein tyrosine phosphatase was shown to activate the cyclin-dependent kinases and is thus involved in regulation of the cell cycle (Gautier et al., 1991). The cyclin-dependent kinase interacter 1 molecule (Cdi1) is a dual specificity protein tyrosine phosphatase that was shown to form stable complexes with the cyclin-dependent kinases and inhibit progression through the cell cycle (Gyuris et al., 1993).

A number of other dual specificity protein tyrosine phosphatases have been shown to inactivate the mitogen activated kinases by dephosphorylation and these have been renamed as MAP Kinase Phosphatases (MKPs).
Table 1.1: Dual specificity phosphatases catalyze the hydrolysis of phosphate from phosphotyrosyl, phosphoserine, and phosphothreonine residues.

<table>
<thead>
<tr>
<th>Dual Specificity Phosphatases</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdc25A</td>
<td>(Wickramasinghe et al., 1995)</td>
</tr>
<tr>
<td>cdc25B</td>
<td>(Baldin et al., 1997)</td>
</tr>
<tr>
<td>cdc25M1</td>
<td>(Nargi and Woodford-Thomas, 1994)</td>
</tr>
<tr>
<td>MKP-1/CL100/hVH1/Erp/3CH134</td>
<td>(Keyse and Emslie, 1992)</td>
</tr>
<tr>
<td>MKP-2/hVH2/TYP-1</td>
<td>(Guan and Butch, 1995)</td>
</tr>
<tr>
<td>MKP-3/Pyst1/rVH6</td>
<td>(Muda et al., 1996; Muda et al., 1996)</td>
</tr>
<tr>
<td>MKP-4</td>
<td>(Muda et al., 1997)</td>
</tr>
<tr>
<td>PAC-1</td>
<td>(Yi et al., 1995)</td>
</tr>
<tr>
<td>VHR</td>
<td>(Ishibashi et al., 1992)</td>
</tr>
<tr>
<td>B59</td>
<td>(Shin et al., 1997)</td>
</tr>
<tr>
<td>hVH3/B23</td>
<td>(Ishibashi et al., 1994)</td>
</tr>
<tr>
<td>PTEN/MMAC1/TEP1</td>
<td>(Li et al., 1997)</td>
</tr>
<tr>
<td>KAP/Cdi</td>
<td>(Gyuris et al., 1993; Hannon et al., 1994)</td>
</tr>
</tbody>
</table>

1.3.2: Low Molecular Weight Phosphatase Family

The low molecular weight protein tyrosine phosphatases are small soluble enzymes that show no sequence similarities to the other members of the PTP superfamily with the exception of the minimal active site motif (CX5R) (Ramponi and Stefani, 1997). These enzymes are under 170 amino acid residues in length and have no known regulatory domains. No physiological role for this subfamily of phosphatases has been described to date. However, overexpression of LMW-PTP resulted in a marked reduction in PDGF-dependent mitogenesis.
(Berti et al., 1994). More recently, it was shown that LMW-PTP overexpression inhibited Src protein tyrosine kinase binding to the PDGF-receptor and abrogated activation of Src in response to PDGF (Chiarugi et al., 1998).

1.3.3: The Phosphotyrosine-Specific Phosphatase Family

This group comprises enzymes having strict specificity for protein tyrosine residues (Pot and Dixon, 1992). These molecules contain an extended active site motif, (I/V)HCXAGXXR(S/T)G, that defines the members of this PTP family. The essential cysteine and arginine residues are present in this signature motif. A member of this group, PTP-ε, is the subject of this thesis.

These phosphotyrosine-specific protein tyrosine phosphatases, like the PTKs, can be further sub-divided based on the presence (the receptor-like protein tyrosine phosphatases), or absence (variously called the non-receptor-like, cytoplasmic, cytosolic or intracellular protein tyrosine phosphatases) of a transmembrane domain.

Members of this broad group of phosphotyrosine-specific enzymes show major similarities within the catalytic phosphatase domain but are quite divergent in the non-PTP domains. A great deal of diversity exists in the structural motifs present in the intracellular protein tyrosine phosphatases. These various structural motifs may be involved in protein localization, protein-protein interactions, and regulation of the catalytic activity.
Figure 1.3: Diversity of the protein tyrosine phosphatase superfamily. Many subfamilies exist for both the (A) receptor-like and (B) intacellular PTPs.
1.4: The Phosphotyrosine-Specific Phosphatase Family

1.4.1: Intracellular Tyrosine Phosphatases

The intracellular protein tyrosine phosphatases typically contain a single phosphatase domain in addition to one or more flanking domains having regulatory function(s). SHP-1, SHP-2, and Drosophila Csw, for example, are related phosphatases containing two src-homology-2 (SH-2) domains followed by a single phosphatase domain; PTP-MEG is the prototype for a class of protein tyrosine phosphatases that contain a region of homology with Band4.1 proteins including ezrin and talin; and PTP-PEST and PEP are distinct molecules each containing the Ser/Thr, Pro, Asp/Glu rich PEST domain in their C-terminal segments.

PTP-1B, the first member of this group identified (Tonks et al., 1988) and SHP1/SHP2 illustrates the modular nature of this family. The 35 amino acid carboxy terminal region of PTP-1B localizes the molecule to the endoplasmic reticulum so that the catalytic domain remains on the cytosolic face (Frangioni et al., 1992). Further, PTP-1B is phosphorylated by the epidermal growth factor receptor after associating with it (Liu and Chernoff, 1997). Phosphorylation of tyrosine residue 66 of PTP-1B creates a site that conforms to the SH2 binding site
for the Grb-2 adapter molecule. The C-terminal proline rich region of PTP-1B was shown to associate with the SH3 domain of p130cas (Liu et al., 1996).

SHP1 and SHP2 contain tandem SH2 domains that mediate interaction of these PTPs with a diverse number of phospho-proteins including cytokine receptors and receptor tyrosine kinases (Neel, 1993; Imboden and Koretsky, 1995). SHP-1 acts as a negative regulator for T- and B-cell antigen receptors (Frearson and Alexander, 1997; Ulyanova et al., 1997). SHP-1 associates with both platelet-derived growth factor receptor and the p85 subunit of phosphatidylinositol 3-kinase and might negatively regulate PDGF receptor-mediated signaling in MCF-7 and TRMP cells (Yu et al., 1998). Recently, evidence has been obtained that indicates that one of SHP-1 functions include the positive regulation of Src kinase activity (Somani et al., 1997). SHP-2, can be either a positive or negative regulator of signal transduction depending on the particular cellular context.
Table 1.2: Numerous intracellular tyrosine phosphatases have been identified.

<table>
<thead>
<tr>
<th>Phosphatase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTP-1B/PTP-HA2/PTP1</td>
<td>(Brown-Shimer et al., 1990; Chernoff et al., 1990; Guan et al., 1990)</td>
</tr>
<tr>
<td>TCPTP/PTP-S</td>
<td>(Cool et al., 1989; Swarup et al., 1991; Kamatkar et al., 1996)</td>
</tr>
<tr>
<td>SHP-1/HCP/PTP1C/PTPN6</td>
<td>(Yi et al., 1992)</td>
</tr>
<tr>
<td>SHP-2/SYP/SHPTP2/SHPTP3/PTP2C/PTP1D</td>
<td>(Adachi et al., 1992; Bastien et al., 1993)</td>
</tr>
<tr>
<td>PTP-BAS/hPTP-1E/PTPL1/FAP</td>
<td>(Banville et al., 1994; Maekawa et al., 1994; Saras et al., 1994)</td>
</tr>
<tr>
<td>PTP-BL/RIP</td>
<td>(Hendriks et al., 1995)</td>
</tr>
<tr>
<td>PTP-RL10</td>
<td>(Higashitsuji et al., 1995)</td>
</tr>
<tr>
<td>HePTP/LC-PTP</td>
<td>(Adachi et al., 1992; Zanke et al., 1992)</td>
</tr>
<tr>
<td>PCPTP1</td>
<td>(Shiozuka et al., 1995)</td>
</tr>
<tr>
<td>PTP3</td>
<td>(Gamper et al., 1996)</td>
</tr>
<tr>
<td>STEP</td>
<td>(Lombroso et al., 1991; Li et al., 1995; Bult et al., 1997)</td>
</tr>
<tr>
<td>PTP-PEST/P19-PTP</td>
<td>[Yang, 1993 #454; den Hertog, 1992 #793]</td>
</tr>
<tr>
<td>PTP-K1</td>
<td>(Huang et al., 1996)</td>
</tr>
<tr>
<td>BDP1</td>
<td>(Kim et al., 1996)</td>
</tr>
<tr>
<td>PTPG1</td>
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</tr>
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<td>PTP20</td>
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<td>pez</td>
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<td>PTP36</td>
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<td>PRL-1</td>
<td>(Diamond et al., 1994)</td>
</tr>
<tr>
<td>PTP(CAAX1/2)</td>
<td>(Cates et al., 1996)</td>
</tr>
<tr>
<td>FLP1,PTP-HSC</td>
<td>(Cheng et al., 1996; Dosil et al., 1996)</td>
</tr>
</tbody>
</table>
1.4.2: Receptor-Like Protein Tyrosine Phosphatases

Receptor-like protein tyrosine phosphatases make up the second group of tyrosine-specific phosphatases (Lau and Baylink, 1993; Walton and Dixon, 1993). These molecules are type I membrane spanning proteins with a single transmembrane domain separating a large variety of N-terminal extracellular domains from the C-terminal cytoplasmic phosphatase domain(s). The receptor-like protein tyrosine phosphatases contain either a single (as in the case of HPTP-β) or two tandemly repeated phosphatase domains with the latter configuration being the most common.

1.4.3: Diversity of Extracellular Domains in RPTPs

The complexity of extracellular domains in the receptor-like protein tyrosine phosphatases presumably reflects the diversity of functions for these molecules. A wide variety of motifs common to other transmembrane-containing or extracellular proteins have been found in the ectodomain of receptor-like protein tyrosine phosphatases. The roles, if any, that these extracellular domains play in regulating phosphatase functions is poorly understood.

Extracellular domains range from being extremely small with no probable function as in the case of the seven amino acids long extracellular domain of
mouse PTP-Φ, or can be extremely large as in the case of CD45 and LAR. These include fibronectin type III (FNIII) motifs, disulphide-linked immunoglobulin (Ig) folds, and others (see below). Single or multiple repeats of 90-100 residue long fibronectin type III (FNIII) motifs in the extracellular domain are common and exist alone (as for the 16 FNIII repeats in DEP-1) or in combination with other motifs. Numerous protein tyrosine phosphatases exhibit motifs having the disulphide-linked immunoglobulin (Ig) fold. The extracellular domain of LAR, for example, contains three N-terminal Ig folds, followed by eight FNIII motifs.

The 170 amino acid MAM domain was defined as a region of homology to meprin A and B, A5 glycoprotein and RPTP-μ. The MAM domain exists in the N-terminus of RPTP-κ and PCP-2 (Wang et al., 1996). The extracellular MAM domain of RPTP-μ and RPTP-κ is essential for homophilic cell-cell interaction (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994; Zondag et al., 1995). The C-terminal non-catalytic carbonic anhydrase (CAH) homology region followed by a single FNIII motif attached to a long cysteine-free spacer region occurs in the extracellular domain of RPTP-β/ζ (Krueger and Saito, 1992) and RPTP-γ (Barnea et al., 1993). The CAH domain of RPTP-β/ζ was recently shown to be a functional ligand for the neuronal receptor contactin (Peles et al., 1995).

RPTP-α and PTP-ε, the subject of this thesis, contain very small, yet unrelated, glycosylated extracellular domains (123 amino acids for RPTP-α and 27 amino acids for PTP-ε) with no known signature motif (Saito, 1993). The extracellular domain of CD45 consists of a large cysteine rich region adjacent to
the transmembrane domain with an N-terminal segment expressed as a variety of isoforms due to the alternate splicing of three exons (Okumura et al., 1996).

Table 1.3: Diversity of Receptor-like Protein Tyrosine Phosphatases

<table>
<thead>
<tr>
<th>Phosphatase</th>
<th>Extracellular Domain Motif</th>
<th>PTP Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>unique</td>
<td>2</td>
</tr>
<tr>
<td>LAR</td>
<td>3 Ig 9 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>LAR-PTP</td>
<td>3 Ig 8 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>PTPσ</td>
<td>3 Ig 8 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>PTPθ</td>
<td>3 Ig 9 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>DLR</td>
<td>3 Ig 10 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>DPTP</td>
<td>2 Ig 2 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>CRYα</td>
<td>3 Ig 4/8 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>PTP-P1/PS</td>
<td>2 Ig 4 FNIII</td>
<td>2/1</td>
</tr>
<tr>
<td>DPTP69D</td>
<td>FNIII</td>
<td>2</td>
</tr>
<tr>
<td>DPTP99A</td>
<td>4 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>DPTP10D</td>
<td>8 FNIII</td>
<td>1</td>
</tr>
<tr>
<td>DPTP4E</td>
<td>11 FNIII</td>
<td>1</td>
</tr>
<tr>
<td>OST-PTP</td>
<td>10 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>HPTPβ</td>
<td>16 FNIII</td>
<td>1</td>
</tr>
<tr>
<td>PTP-U2</td>
<td>8 FNIII</td>
<td>1</td>
</tr>
<tr>
<td>Byp</td>
<td>8 FNIII</td>
<td>1</td>
</tr>
<tr>
<td>DÉP1</td>
<td>8 FNIII</td>
<td>1</td>
</tr>
<tr>
<td>SAP1</td>
<td>8 FNIII</td>
<td>1</td>
</tr>
<tr>
<td>GLEPP1,PTP-U1</td>
<td>8 FNIII</td>
<td>1</td>
</tr>
<tr>
<td>RPTP-BK</td>
<td>7 FNIII</td>
<td>1</td>
</tr>
<tr>
<td>PTP NU-3</td>
<td>9 FNIII</td>
<td>2</td>
</tr>
<tr>
<td>PTPα</td>
<td>127aa</td>
<td>2</td>
</tr>
<tr>
<td>PTPε</td>
<td>27aa</td>
<td>2</td>
</tr>
<tr>
<td>PTPα</td>
<td>MAM Ig FNIII</td>
<td>2</td>
</tr>
<tr>
<td>PTPκ</td>
<td>MAM Ig FNIII</td>
<td>2</td>
</tr>
<tr>
<td>PFC2</td>
<td>MAM Ig FNIII</td>
<td>2</td>
</tr>
<tr>
<td>PTPα</td>
<td>MAM Ig FNIII</td>
<td>2</td>
</tr>
<tr>
<td>RPTP psi</td>
<td>MAM Ig FNIII</td>
<td>2</td>
</tr>
<tr>
<td>PTP-oc</td>
<td>8aa</td>
<td>1</td>
</tr>
<tr>
<td>PTPα/β</td>
<td>CA, 1 FNIII, spacer</td>
<td>2</td>
</tr>
<tr>
<td>PTPγ</td>
<td>CA, 1 FNIII, spacer</td>
<td>2</td>
</tr>
<tr>
<td>PTP-SL</td>
<td>83aa</td>
<td>1</td>
</tr>
<tr>
<td>PTP-BR7</td>
<td>202aa</td>
<td>1</td>
</tr>
<tr>
<td>ChPTP</td>
<td>S/T/P, Sp, FNIII</td>
<td>2</td>
</tr>
<tr>
<td>PTP-1AR</td>
<td>RGDS, 614aa</td>
<td>1</td>
</tr>
<tr>
<td>Phogrin</td>
<td>C rich</td>
<td>1</td>
</tr>
<tr>
<td>PTP-1A2</td>
<td>C rich</td>
<td>2</td>
</tr>
<tr>
<td>PTP-NP</td>
<td>C rich</td>
<td>1</td>
</tr>
</tbody>
</table>

1.4.4: Catalytic Properties and Structure of Protein Tyrosine Phosphatases

Although protein tyrosine phosphatase activities had been previously studied in a variety of cell extracts (Hunter, 1989; Tonks and Charbonneau, 1989), thorough understanding of the catalytic properties of protein tyrosine phosphatases has advanced significantly since the isolation and characterization of the first protein tyrosine phosphatase, PTP-1B (Tonks et al., 1988; Tonks et al., 1988). Charbonneau (1989) determined the partial amino acid sequence of the soluble 35kd PTP-1B purified from human placenta. A 240 amino acid region of PTP-1B showed similarity to the intracellular tandemly duplicated region of the previously cloned leukocyte cell surface protein, CD45 (Charbonneau et al., 1988). CD45 was subsequently shown to have intrinsic PTPase activity (Tonks et al., 1988). In this way the catalytic region of protein tyrosine phosphatases was first defined.

Protein tyrosine phosphatases catalyze the hydrolysis of the phosphoester bond of phosphotyrosyl residues on proteins (Zhang and Dixon, 1994; Barford, 1995). PTP catalysis requires no cofactors or metal ions. The highly conserved cysteine in the CX5R active site motif is essential for the catalytic activity of these enzymes and acts as a nucleophile by accepting the phosphate moiety from the phosphorylated tyrosine residue. In this way a phospho-cysteine enzyme intermediate is created. The arginine in the CX5R active site motif is required to stabilize the transition-state. Transfer of the phosphate moiety from the cysteine to a water molecule regenerates the enzyme.
Figure 1.4: Proposed mechanism of protein tyrosine phosphatase hydrolysis of phosphotyrosyl residues (Adapted from Dixon 1995).

The crystal structure of the catalytic domain of a number of protein tyrosine phosphatases has been determined to date (Barford, 1995; Bliska, 1995; Jia, 1997). The structures of the membrane proximal catalytic domains of receptor-like human RPTP-α was determined at a resolution of 3.2 angstroms (Bilwes et al., 1996) as was that of human PTP-μ (Hoffmann et al., 1997). The crystal structure of the catalytic domain of human cytoplasmic phosphatase PTP-1B was determined alone (Barford et al., 1994) as well as that of the catalytic cysteine mutagenized to serine to enable crystal formation in the presence of a phosphopeptide (Jia et al., 1995). The crystal structure of the catalytic domain of Yersinia PTP in complex with tungstate was also determined (Stuckey et al., 1994). Most recently, the crystal structure of all but the last 66 residues of the intracellular phosphatase SHP-2 was determined (Hof et al., 1998).
Figure 1.5: A ribbon diagram representing the crystal structure of the first catalytic domain of RPTP-α. A) Side view of two molecules of RPTP-α. B) Top view showing interaction of the wedge region with the active site (AS) of the opposing molecule. Images were generated, using RasMol software, from the crystal coordinates of RPTP-α available from the Brookhaven Protein Database.
In all cases the active site forms the base of a cleft in which active site residues coordinate the binding of the phosphotyrosyl residue of the phosphopeptide substrate near the catalytically essential cysteine residue in a position that allows this residue to function as a nucleophile.

Investigation of the crystal structure of RPTP-α has lead to the hypotheses that protein-protein interactions may regulate enzyme activity. Bilwes et al. (1996) proposed that symmetrical dimerization of two molecules of RPTP-α leads to active-site blockage of one molecule by a membrane proximal 'wedge' of the opposing molecule and that this is a physiologically important mechanism for maintaining low basal catalytic activity of these enzyme. This idea is further supported through in vitro studies of a mutated EGFR-CD45 chimera (Majeti et al., 1998). However, this mechanism of negative regulation is likely not a general one for receptor-like protein tyrosine phosphatases as no evidence was obtained for it in the determination of the human PTP-μ crystal structure (Hoffmann et al., 1997).

The recent structural determination of SHP-2 indicated that the active site of the PTP domain was blocked by the N-terminal SH2 domain in such a fashion that both SH2 domain-phosphotyrosyl binding pockets were exposed to the solvent (Hof et al., 1998). This blockage suggests that SHP-2 is inactive in this closed conformation and is further supported by biochemical analysis of this molecule (Dechert et al., 1994).
1.4.5: Alternative Splicing of Protein Tyrosine Phosphatases as a Means of Diversification

Many protein tyrosine phosphatases exhibit structural diversification through the use of alternative splicing of various exons. For example, the STEP family of protein tyrosine phosphatases is enriched within the central nervous system and includes transmembrane, as well as soluble, cytosolic proteins derived from alternate splicing (Bult et al., 1997). In the case of receptor-like protein tyrosine phosphatases the extracellular domain may show diversity due to alternative splicing, as in the case of CD45 in which exons 4, 5 and 6 can be differentially spliced. Alternative splicing can also result in the creation of isoforms that have either one or two phosphatase domains. The first example is with the double phosphatase domains of PTP-P1 and its single domain isoform PTP-PS (Pan et al., 1993). Alternative splicing of PTP-S2 and PTP-S4 gives rise to two protein-tyrosine phosphatases with distinct substrate specificities and subcellular locations (Kamatkar et al., 1996). Numerous isoforms of LAR that are preferentially expressed and developmentally regulated have been identified in the nervous system (Zhang and Longo, 1995).

Alternative splicing results in the creation of different isoforms that may have distinct distribution of expression in tissues and potentially distinct functional roles to play in signal transduction events. This thesis revolves around the genetic analysis of PTP-ε and this molecule exists as two alternately spliced isoforms as detailed below.
1.4.6: Substrates of Protein Tyrosine Phosphatases

Unlike the protein tyrosine kinases, very little information on the \textit{in vivo} substrate specificity of the various protein tyrosine phosphatase family members has been obtained. This is predominantly due to the technical difficulty in following the removal of phosphate from proteins as opposed to its addition. A number of reports have investigated the activity and specificity of phosphatases for \textit{in vitro} peptide substrates (Tonks \textit{et al.}, 1990; Ramachandran \textit{et al.}, 1992; Cho \textit{et al.}, 1993; Dechert \textit{et al.}, 1994; Wu \textit{et al.}, 1997).

For example, to compare the substrate specificity and function of CD45 and RTPP-\(\alpha\), RTPP-\(\alpha\) was overexpressed in a CD45-deficient T-lymphoma cell (Ng \textit{et al.}, 1997). These authors showed that high levels of RTPP-\(\alpha\) expression did not fully restore T cell receptor (TCR)-mediated signaling. RTPP-\(\alpha\) was not able to reconstitute the phosphorylation of the CD3-zeta chain. Nor was it able to increase expression, upon TCR-stimulation, of the activation marker, CD69. RTPP-\(\alpha\) was not able to significantly alter the phosphorylation state or kinase activity of two CD45 substrates, lck or fyn. All of this data suggests that CD45 and RTPP-\(\alpha\) do not have the same specificity or function in T-cells or that the extracellular and transmembrane domains of the two molecules may have a role in localization of these PTPs.

Recently, a novel strategy that involves the creation of "substrate-trapping" mutant forms of a protein tyrosine phosphatase has been developed (Garton \textit{et al.}, 1996). The mutant PTP lacks catalytic activity but retains the ability to bind substrates. Application of this technique to PTP-PEST found that
the mutant substrate-trapping form of PTP-PEST forms stable complexes with p130<sup>cas</sup>, in lysates from several cell lines and in transfected cells. This association was not observed with other PTP family members, suggesting that p130<sup>cas</sup> represents a physiologically relevant substrate for PTP-PEST. Flint (1997) created a mutant "substrate-trapping" form of PTP-1B by mutation of the invariant catalytic acid (Asp-181). Expression of this PTP-1B<sup>D181A</sup> mutant in COS and 293 cells resulted in an enzyme that competed with the endogenous phosphatase for substrates. Phosphotyrosine accumulated on the epidermal growth factor-receptor and on other proteins of 120, 80, and 70 kDa. The association between the PTP-1B<sup>D181A</sup> mutant and these substrates was sufficiently stable to permit isolation of the complex by immunoprecipitation. This suggests that the EGF receptor is an <i>in vivo</i> substrate for PTP1B.

By using GST-TCPTP fusion proteins for affinity chromatography, three cytoplasmic proteins of 120, 116, and 97 kDa that interact with TCPTP have been isolated (Tiganis <i>et al.</i>, 1997). Analysis of p97 identified it as the nuclear import factor p97 (Importin-β), an essential component of the nuclear import machinery. Technique, such as these, can be adapted for identification of substrates of other protein tyrosine phosphatases.
1.5: Protein Tyrosine Phosphatase-Epsilon

1.5.1: PTP-ε Exists as Two Isoforms

This thesis revolves around a genetic analysis of protein tyrosine phosphatase epsilon (PTP-ε). The complete cDNA for the receptor-like isoform of PTP-ε was first cloned by Krueger et al. (1990) from a human placenta cDNA library by low stringency hybridization with an unrelated Drosophila phosphatase cDNA clone.

PTP-ε was initially identified as a type I receptor-like transmembrane protein of 699 amino acid residues in length (Krueger et al., 1990). The intracellular portion of the protein is composed of a short juxtamembrane region followed by two tandemly duplicated phosphatase domains that are separated by a short inter-catalytic spacer of 48 amino acids. The extracellular domain of PTP-ε is small at 27 amino acids, after cleavage of the 20 amino acid signal sequence. It contains two potential N-linked glycosylation sites and its amino acid sequence shows no similarity to any known protein. The function of the extracellular domain is unknown.

Recently, Elson and Leder (1995) identified a non-transmembrane-containing isoform of PTP-ε from both mouse and human mRNA. This isoform lacked the extracellular region as well as the transmembrane domain. The cytoplasmic isoform has a unique twelve amino acid N-terminal region and a distinct 5' untranslated region. However, the entire cytoplasmic region of the
transmembrane isoform is identical to that of the cytoplasmic isoform. Nakamura (1996) cloned both the cytoplasmic and transmembrane isoforms from rat spleen RNA.

### Transmembrane Isoform

<table>
<thead>
<tr>
<th>Signal Sequence</th>
<th>Extracellular Domain</th>
<th>Transmembrane Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDP&lt;sub&gt;6&lt;/sub&gt;QGLASVSLARAGQNDFTPTESWTSTTTAQPDPFQASQ</td>
<td>ELLAVQHELQGLLDELLAAVFR</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Juxtamembrane Domain</th>
</tr>
</thead>
</table>
| CHO | CHO |}

### Cytoplasmic Isoform

<table>
<thead>
<tr>
<th>PRKQRKAVVSNDKMFNGITESQ</th>
</tr>
</thead>
</table>

Figure 1.6: PTP-ε exists as two isoforms, a receptor-like PTP having a short extracellular domain and an intracellular PTP having a unique twelve amino acid N-terminal region. PTPase D1 and D2 define the first and second phosphatase domains.
The existence of the distinct 5' untranslated region between the receptor-like form and the intracellular form as well as their predominantly non-overlapping expression patterns (see below) suggest that the two isoforms may be regulated from separate promoters (Elson et al., 1996). However, as the complete Ptpre genomic structure has not been determined, this has yet to be confirmed.

The Ptpre gene maps to mouse chromosome 7 and human chromosome 10q26 (van den Maagdenberg et al., 1995; Elson et al., 1996; Melhado et al., 1996). The mapping of Ptpre to a single locus in both mouse and human supports the hypothesis that the receptor-like and cytoplasmic isoforms of PTP-ε arise from a single gene through the use of alternative promoters and 5' exons. The gene encoding the closely related PTP, RPTP-α, has been assigned to human chromosome 20p13 (Jirik et al., 1992) as well as to rat chromosome 3q36 and mouse chromosome 2G (Saadat et al., 1995).

1.5.2: PTP-ε is Closely Related to RPTP-α

The intracellular portion of protein tyrosine phosphatase-epsilon is highly related to the receptor-like protein tyrosine phosphatase-alpha (RPTP-α) showing 78% amino acid identity within phosphatase domain 1 and 74% identity within phosphatase domain 2 (Figure 1.6). The inter-catalytic domain and the intracellular region proximal to the transmembrane domain (i.e. the
juxtamembrane region) each shows approximately 52% amino acid identity. The extracellular domains are not conserved between these two proteins.

RPTP-α is a protein of 793 amino acids that was originally cloned by a number of groups (Jirik et al., 1990; Kaplan et al., 1990; Krueger et al., 1990; Sap et al., 1990). Like PTP-ε, RPTP-α contains a relatively short extracellular domain of 123 amino acid residues in length and a transmembrane domain followed by two cytosolic PTP domains.

Protein tyrosine phosphatase-α is constitutively phosphorylated on the C-terminal tyrosine residue (Tyr789), a consensus binding site (YXNX) for the SH2 domain of the SH3-SH2-SH3 adapter protein GRB2 (den Hertog et al., 1994). These authors showed that RPTP-α, but not a Tyr789->Phe mutant of RPTP-α bound to GRB2 in vitro and that RPTP-α co-immunoprecipitated with GRB2 from NIH 3T3 cells, demonstrating that GRB2 bound to RPTP-α. They were not able to detect the GRB2-SH3-domain binding Son of Sevenless (SOS) molecule, a guanine nucleotide releasing factor for the Ras GTPase, in RPTP-α immunoprecipitates. More recently, it was reported that the C-terminal SH3 domain of GRB2 is also involved in binding to RPTP-α in vitro (den Hertog and Hunter, 1996). The site of interaction of the C-terminal SH3 domain of GRB2 with RPTP-α was mapped using deletion mutants to an 18-residue region in the first phosphatase domain. The role of Grb-2 association to RPTP-α is unknown.
Figure 1.7: CLUSTAL V multiple sequence alignment of murine PTP-ε (top) and murine RPTP-α (bottom). Identical residues are indicated by an asterisk (*) and conserved residues are indicated by a dot (.).
1.5.3: PTP-ε Isoforms Shows Restricted Expression Patterns

Unlike RPTP-α, which is widely expressed in diverse tissues and cell lines (Jirik et al., 1990; Kaplan et al., 1990; Sap et al., 1990), the two isoforms of PTP-ε exhibit restricted tissue specific and non-overlapping expression patterns (Elson and Leder, 1995). PTP-ε was found to be highly expressed in murine brain and testis (Yi et al., 1991). Transcripts were also detected in fibroblasts, myeloid cells, macrophages and T cells. In contrast, neither PTP-ε transcripts nor protein were detected by Northern analysis and Western analysis in a panel of mouse and human B-cell lines (Ian Melhaldo, personal communication). The brain and testis transcripts were later identified to be those of the transmembrane isoform (Elson and Leder, 1995). Relatively high levels of the mRNA for the transmembrane isoform expressed in the lung while heart shows lower levels of this mRNA. Not all tissues have been examined for PTP-ε expression. For example, the expression status of PTP-ε in the gastrointestinal tissues, tissues having cells that undergo differentiation in the adult, has yet to be determined.

Elson and Leder (1995) also showed that the transmembrane isoform is very highly expressed in experimental murine mammary tumors initiated by \( c-neu \) and \( v-Ha-ras \), but not in tumors initiated by \( c-myc \) or \( int-2 \). These authors suggest that PTP-ε may play a role in \( ras- \) and \( neu \)-mediated transformation of mammary epithelium. However, transgenic mice over-expressing the transmembrane isoform in mammary tissues did not develop tumors nor was there a change in latency of tumor formation when crossed on to mice expressing \( c-neu \) or \( v-Ha-ras \) in mammary tissue (A. Elson, personal communication). This suggests that PTP-ε is not causative in tumor formation.
Kaneko (1993), using a RT/PCR derived murine probe for PTP-ε, showed that expression occurs predominantly in Sertoli cells when assayed by Northern blot analysis and by in situ hybridization of normal testis of an adult mouse. This suggests a role for PTP-ε in the maintenance of spermatogenesis. Hendriks (1994) showed by a dot blot strategy that PTP-ε was expressed in embryonic stem cells, embryonic brain (E13.5), neonatal brain and a 15 day old mouse brain as well as in the human ovarian carcinoma cell line OVACAR4.

PTP-ε transcripts are highly expressed in osteoclastic cells generated by coculturing mouse bone marrow cells with mouse calvaria osteoblasts (Schmidt et al., 1996) and in highly enriched mammalian mononucleated osteoclast precursors (Wesolowski et al., 1995).

In situ hybridization detected PTP-ε transcripts in the neural tube of day 12 postcoitum embryos, and in the brain, spinal cord, and ganglions in a ubiquitous manner in late gestational stages (Mukouyama et al., 1997). In 4-day-old neonatal mice, the transcripts were widely distributed in the brain with the highest expression detected in the hippocampus, cerebral cortex, and olfactory bulb. Further, in day 7 and 16 neonatal brains, the strongest PTP-ε gene expression is found in the granular cells of the cerebellum.

The restricted expression pattern of the two isoforms of PTP-ε contrasts with that of RPTP-α which is expressed in all tissues (Jirik et al., 1990; Sap et al., 1990). This suggests that these two related molecules have distinct roles in various cell types.
PTP-ε mRNA levels are increased in a variety of models of cell differentiation. For example, differentiation of mouse erythroleukemia cells with dimethyl sulfoxide results in an initial reduction in PTP-ε mRNA from basal levels followed by an increase that peaked at 18-24 hours after stimulation (Kume et al., 1994). Retinoic acid induced differentiation of mouse embryonal carcinoma (F9) cells resulted in an increased mRNA levels of PTP-ε 48hrs after stimulation (Tsuneizumi et al., 1994). This increase was not observed in differentiation-defective mutant F9 cells.

Human promyeloblastic leukemia HL60 cells, when induced to differentiate to monocyte/macrophage cells with phorbol ester, 12-o-tetradecanoylphorbol-13-acetate (TPA), upregulate both the mRNA and the protein for the cytoplasmic isoform (Elson and Leder, 1995), Melhado, et al. in preparation). The human monoblastoid leukemia U937 cells also differentiate to monocyte- and macrophage-like cells upon treatment with TPA whereas a TPA-resistant U937 variant cell line, UT16, showed altered binding activity of AP-1 complexes, decreased ability to induce c-jun and c-fos gene expression, and failure to differentiate to a monocytic lineage (Seimiya et al., 1995). Most of TPA-inducible tyrosine phosphatase transcripts in U937 cells (including PTP-1C, PTP-MEG2, P19-PTP, PTP-U1, and PTP-ε) were not induced by TPA treatment of UT16 cells.

The *in vitro* differentiation of the rat pheochromocytoma PC12D cell line into neuronal-like cells by stimulation with NGF or FGF resulted in transient
induction of PTP-ε transcripts at a time between the appearance of immediate-
early gene transcripts and transcripts for neuronal cell-specific markers
(Mukouyama et al., 1997).

Differentiation of the rat CG4 cell line to oligodendrocytes led to the
detection, by a differential display strategy, of eleven different protein tyrosine
phosphatases (Ranjan and Hudson, 1996). Most of the phosphatases examined,
including RPTP-α, PTP-ζ, PTP-σ, and PTP-γ, showed an increase in their mRNA
levels during differentiation, with a striking upregulation observed for PTP-ε.

The significance of the upregulation of PTP-ε mRNA in all of these model
systems is unknown. However, it suggests that PTP-ε may have an important
role in development of various tissues.

1.5.5: PTP-α and PTP-ε Negatively Regulate Insulin Receptor Activity In Vitro

With the aim of identifying protein tyrosine phosphatases that negatively
regulate insulin-receptor kinase activity, Moller et al. (1995) developed an in vivo
system using baby hamster kidney (BHK) cells overexpressing the insulin
receptor (BHK-IR). By independently transfecting into the BHK-IR cells
expression plasmids containing cDNAs for six different receptor-like protein
tyrosine phosphatases and six different intracellular protein tyrosine
phosphatases and looking for an abrogation of insulin dependent loss of
adhesion, these authors were able to demonstrate that RPTP-α, PTP-ε and, to a
lesser extent, TCPTP negatively regulated insulin receptor activity in the BHK-IR cell system. In contrast, LAR, PTP-σ, PTP-μ, CD45 and a group of intracellular protein tyrosine phosphatases could not inhibit insulin receptor activity when overexpressed in the BHK-IR cells. Subsequent to this it was shown that RPTP-α preferentially dephosphorylates the β-subunit of the insulin receptor at the cell surface in this BHK-IR cell system (Lammers et al., 1997). These results raise the possibility that PTP-α and PTP-ε might be involved in regulating IR function in vivo. However, as PTP-α was also able to regulate cell-substratum adhesion and EGF-induced A431 cell detachment, it is unlikely that PTP-α is involved solely in the regulation of glucose metabolism (Harder et al., submitted).
1.6: Mice with Natural or Designed Mutations in Various Protein Tyrosine Phosphatases

To date, the genes for a number of protein tyrosine phosphatases, both receptor-like and intracellular, have been disrupted by gene targeting in mice using either conventional gene targeting strategies or a genetrap approach.

CD45 was the first PTP gene to be targeted for disruption in mice. Using the classical approach, (Kishihara et al., 1993) removed the isoform specific exon 6 from the mouse genome. This caused the loss of CD45 expression in B cells and most T cells. This resulted in loss of antigen receptor cross linking-induced cell proliferation in spite of apparently normal B cell development, as well as a block in thymocyte maturation from the double positive (CD4+CD8+) to the single positive (CD4+CD8- and CD4-CD8+) cells. The strategy for targeting CD45 by these authors did not result in a true 'null' genotype. Byth et al. (1996) more recently created a true null phenotype of the CD45 tyrosine phosphatase by insertion of the Neo cassette into exon 9, an exon common to all isoforms of CD45. The spleens from these CD45-null mice contain approximately twice the number of B cells due to the specific expansion of two IgM-high B cell subpopulations and one fifth the number of T cells found in normal controls. These results suggest that CD45 is an important regulator of signal transduction via the TCR complex at multiple stages of T cell development.

TCPTP is an intracellular tyrosine phosphatases expressed in hematopoietic cells (Cool et al., 1989). TCPTP-deficient mutant mice die by 3-5 weeks of age, displaying runting, splenomegaly, and lymphadenopathy.
Interestingly, the bone marrow defect does not appear to be due to a stem cell
defect, but rather to a stromal cell deficiency (You-Ten et al., 1997). Bone marrow
myelopoiesis and thymic T cell development were not significantly affected in
these TCPTP-deficient mice.

The homozygous recessive motheaten (me/me) allele was first
characterized in 1975 and mapped to chromosome 6 in a genetic cross (Green
and Shultz, 1975). The me/me defect involved severe multiple inmunological
abnormalities in essentially all hematopoietic compartments. These mice died
post-natally at an average age of three weeks. The homozygous viable
motheaten mice (me\textsuperscript{v}/me\textsuperscript{v}) exhibit the same phenotype as in me/me mice except
they survive post-natally to a mean age of nine weeks (Tsui and Tsui, 1994;
Shultz et al., 1997). The genetic defects for both me and me\textsuperscript{v} were determined to
be in the intracellular SH2-containing tyrosine phosphatase, SHP-1 previously
known as HCP for hematopoietic cell phosphatase (Kozlowski et al., 1993; Shultz
et al., 1993; Tsui et al., 1993). Molecular analysis of the SHP-1 locus in these mice
indicated that the defects were due to, in the case of the me/me allele, a point
mutation that leads to a 101bp deletion from the first SH2 domain of the spliced
transcript resulting in a frameshift mutation causing a premature termination
and, in the case of the me\textsuperscript{v}/me\textsuperscript{v} allele, a single point mutation results in the
creation of two mutant alternate transcripts.

Yu et al. (1996) bred homozygous, double-mutant me\textsuperscript{v}/me\textsuperscript{v} x
recombination activation gene-1 (RAG-1\textsuperscript{-/-}) mice and found inflamed paws, and
splenomegaly with elevated myelopoiesis. The recombination activation gene-1
mutant disrupts V(D)J rearrangement and thus results in impaired development
of B and T cells. These double knockouts indicate that the pleiotropic motheaten phenotype is not due to B and T cell immune-dysfunction alone.

Skarnes et al. (1995) generated, in embryonic stem cells, mutant βgeo fusion proteins of two receptor-like protein tyrosine phosphatase genes, LAR and PTPk, in a "secretory trap" modification of the gene trap strategy. Where the βgeo cassette is a fusion of the LacZ gene and the neomycin resistance gene. Germline transmission of these mutant ES cells allowed the authors to cross mutant mice for each of these genes to homozygosity. This resulted in mice that were viable and fertile. No further characterization of these mutant mice has been published to date.

Using the classical gene targeting strategy with the aim of knocking out the LAR protein tyrosine phosphatase, (Schaapveld et al., 1997) created mutant mice having a truncated LAR that lacked the cytoplasmic tyrosine phosphatase domains. These LAR mutant mice developed and grew normally and exhibited no overt histological tissue abnormalities. Mammary glands of LAR mutant females, however, were incapable of delivering milk due to an impaired terminal differentiation of alveoli at late pregnancy. Interestingly, this phenotype had variable penetrance depending on the background strain to which the mutation was bred. The 129/Sv x C57Bl/6 strain containing this modified locus exhibited approximately 50% penetrance in contrast to the C57Bl/6 strain that exhibited 100% penetrance. This suggests that LAR function is modified by one or more other unknown loci.
1.7: Genetic Modification of the Mouse Genome

Alteration of the genome of experimental organisms by various genetics techniques has provided valuable insight into the genetic of development, behavior, cellular function, physiology, immunology and disease. The rapidly breeding invertebrates, *Drosophila melanogaster* and *Caenorhabditis elegans* as model organisms, have laid foundations for the study of genetics of eukaryotes. The lessons derived from the study of these model organisms can be translated into the genetic understanding of mammalian biology and disease. However, the greater complexity of the mammalian organism has required the development of methodologies that permit the analysis of mammalian genetics. The mouse model system has been studied, through the use of selective breeding and the identification of random or mutagen-induced mutants for over a century.

1.7.1: Creation of Transgenic Mice

The introduction of novel genetic elements into the genome results in the creation of transgenic organisms. The work of the collaborating laboratories of Richard Palmiter and Ralph Brinster led to the development of protocols for the production of transgenic mice by the introduction of DNA by microinjection directly into the pro-nucleus of newly fertilized mouse embryos and the subsequent implantation of the transgenic embryos into the oviduct of pseudopregnant female recipients (Brinster *et al.*, 1981). Implementation of these
landmark protocols in many laboratories around the world has led to a greater understanding of mammalian biology.

1.7.2: Classical Gene Targeting

A major advance in the field of mouse genetics was the creation of locus-specific mutant alleles by targeted disruption in mouse embryonic stem cells. Thomas et al. (1987) were the first to produce new strains of mice that had their endogenous gene modified in embryonic stem cells by the process of homologous recombination. Creation of homozygous 'null' mutant mice has provided fascinating and sometimes surprising insight into the function and regulation of genes involved in diverse processes from developmental biology to cancer pathology and has also been used to develop mouse models of human disease (Moreadith and Radford, 1997). However, in the event of an embryonic lethal phenotype due to the targeted disruption of a particular gene, the study of the genes function in a mature animal is not possible.

To target a gene by homologous recombination, the genomic structure must first be cloned and characterized and a targeting vector created in which a critical exon(s) of the gene is replaced or disrupted, for example, by a selectable marker. In order to maximize the frequency of homologous recombination it is important that the gene of interest be isolated from a genomic library that is isogenic with that of the ES cell genome. The targeting vector must contain substantial lengths (on the order of 4-10 kb or more) of homologous DNA flanking the exon(s) to be targeted. This maximizes the frequency of homologous
recombination with the endogenous locus. Electroporation of the targeting vector into undifferentiated ES cells results in either random (non-homologous) integration or the desired recombination of the vector into the homologous locus. Selection strategies in conjunction with screening using Southern blotting and/or PCR allow the identification of distinct ES cell clones containing the mutant allele. It is of vital importance to minimize the number of passages of the undifferentiated ES cells before and after transfection and selection of mutants, since extended periods in culture will compromise the totipotency of the ES cells, or the ability to "go germline" in other words.

Injection of the mutant ES cell clones into blastocysts, and implantation of these into a pseudo-pregnant female results in chimeric offspring exhibiting variable genetic contribution by the ES cell. Mouse coat color provides a simple strategy for assessing germline contribution. This is typically performed by using an ES cell line derived from a mouse with an agouti coat color, such as the R1 line (Nagy et al., 1993), and blastocysts derived from mice with a black coat color. Offspring in which the ES cell contributes to the germline allow the propagation of the mutant allele through successive generations in a Mendelian fashion. Crossing of two heterozygous mice leads to offspring having a homozygous gene disruption.

Approximately, one third of homozygous knockout mice exhibit an embryonic or peri-natal lethality (TBASE, the Online Transgenic Database). In the event of an embryonic lethal phenotype due to the targeted disruption followed by breeding to homozygousity of a particular gene, the study of that gene's loss of function in a mature animal is impossible. A strategy to overcome this has been developed (see below).
1.7.3: Temporal and Tissue Specific Genetic Ablation

In an attempt to overcome the problem of embryonic lethality, a modification of the classical gene targeting technology has been developed that involves the use of the bacteriophage P1 site specific recombinase Cre and its LoxP target signal sequences recognized by this enzyme (Abremski and Hoess, 1984). This permits total as well as tissue-specific knockouts (Gu et al., 1993; Rajewsky et al., 1996).

The LoxP site is a 34bp stretch of DNA that contains two 13bp inverted repeats separated by an 8bp spacer region (Figure 1.9 a). The site specific recombinase Cre binds as a homodimer to the LoxP DNA sequence in trans to permit recombination with a second Cre-bound LoxP site. Depending on the relative orientation of the two LoxP sites, the Cre recombinase can mediate site-specific DNA deletion or inversion (Figure 1.9 b and c).

The basis of this technique involves the use of LoxP sites flanking, in a fashion that is non-deleterious to the gene expression, the gene or exon(s) to be deleted (Figure 1.9 b). A targeting vector incorporating these LoxP sites flanking the exon of interest is used to replace the wild-type exon in ES cells by homologous recombination. Transfection of a Cre expressing construct into a population of these ES cells permits the result analogous to the conventional targeting strategy, i.e. total ablation of the LoxP flanked exon.
a) The LoxP Sequence

<table>
<thead>
<tr>
<th>Inverted Repeat</th>
<th>Spacer</th>
<th>Inverted Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATAACTTCGTATA</td>
<td>ATGTATGC</td>
<td>TATACGAAGTTAT</td>
</tr>
<tr>
<td>13bp</td>
<td>8bp</td>
<td>13bp</td>
</tr>
</tbody>
</table>

b) Site specific deletion
c) Site specific inversion

Figure 1.8. The site specific recombinase Cre binds the LoxP DNA sequence \textit{in trans} to permit recombination with a second Cre bound LoxP site. A single LoxP site is composed of two 13bp inverted repeats separated by an 8bp spacer region (Abremski and Hoess, 1984).
The pFlox strategy developed by (Gu et al., 1994) involves the use of three \textit{LoxP} sites in which two sites flank the gene or exon(s) of interest and two flank the negative and positive selectable markers - thymidine kinase and neomycin phosphotransferase (Figure 1.10). The initial electroporation of the targeting vector into ES cells permits the creation of homologous recombinant clones. These clones can be further modified in culture by transient transfection of a Cre recombinase-expressing plasmid (Gu et al., 1993). Under the selective pressure of gancyclovir, two excision possibilities exist; i) the Cre enzyme recombines the DNA between the two outer most \textit{LoxP} sites thus giving a type I or total deletion; ii) Cre mediated recombination between the two \textit{LoxP} sites flanking the selectable markers leads to a type II or "floxed" deletion in which two \textit{LoxP} sites remain in the genome flanking the desired exon in a fashion that is non-deleterious to the expression of the floxed gene. The Cre-mediated deletion event is random with respect to the relative ratio of type I vs. type II deletions. Numerous groups using variations on this strategy have obtained a predominance of type I over the required type II deletion, and in some cases no type II deletions were identified. The reason for this variability is unknown and may be dependent on the chromosomal context within the ES cell, or may be due to technical differences used by the various labs.
Figure 1.9. The pFlox gene targeting strategy involves the use of three LoxP sites, of which two flank the selectable markers - HSV-thymidine kinase (Tk) and neomycin phosphotransferase (Neo) and two flank the exon(s) or gene of interest.

1.7.4: Cre Recombinase Expression in Mutant Mice

Cre expression can be introduced after generation of mice homozygous for the floxed exon. A second mouse expressing the Cre recombinase transgene in a tissue specific fashion (as dictated by specific promoter and enhancer regulatory elements) can be mated with the 'floxed' mouse. A proportion of the F1 generation will have both the Cre protein and one allele of the floxed gene. Further breeding and analysis by Southern blot will generate mice having both floxed alleles and the Cre transgene present in the genome. All tissues in which
the Cre protein is expressed will delete the sequences between the two LoxP sites at both alleles thus creating a tissue specific knockout. Many Cre-expressing transgenic mice have been produced to date that express the Cre recombinase in a tissue specific fashion as dictated by specific promoter and enhancer regulatory elements (see Table 1.4).

Table 1.4: Cre recombinase-expressing transgenic mice that have been produced.

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>alphaMyHC-Cre</td>
<td>Cardiac myocytes</td>
<td>(Agah, et al., 1997)</td>
</tr>
<tr>
<td>P0-Cre</td>
<td>Myelinating Schwann Cells</td>
<td>(Akagi, et al., 1995)</td>
</tr>
<tr>
<td>POMC-Cre</td>
<td>Pituitary</td>
<td>(Akagi, et al., 1997)</td>
</tr>
<tr>
<td>IRRP-Cre</td>
<td>Retina</td>
<td>(Akagi, et al., 1997)</td>
</tr>
<tr>
<td>aP2-Cre</td>
<td>Adipose tissue</td>
<td>(Barlow, et al., 1997)</td>
</tr>
<tr>
<td>nestin-cre</td>
<td>for mosaic animals</td>
<td>(Betz, et al., 1996)</td>
</tr>
<tr>
<td>CMV-Cre-ERT</td>
<td>Conditional</td>
<td>(Feil, et al., 1996, Brocard, et al., 1997)</td>
</tr>
<tr>
<td>lck-Cre</td>
<td>T cell lineages</td>
<td>(Hennet, et al., 1995)</td>
</tr>
<tr>
<td>Mx-Cre</td>
<td>INF-responsive tissues</td>
<td>(Kuhn, et al., 1995)</td>
</tr>
<tr>
<td>Ad EIIa-Cre</td>
<td>Zygote</td>
<td>(Lakso, et al., 1996)</td>
</tr>
<tr>
<td>CMV-Cre</td>
<td>deleter strains</td>
<td>(Lasko et al., 1992, Schwenk et al., 1995)</td>
</tr>
<tr>
<td>αA-crystallin-Cre</td>
<td>Lens</td>
<td>(Lasko, et al., 1992)</td>
</tr>
<tr>
<td>βActin-Cre</td>
<td>Early mouse embryo</td>
<td>(Lewandoski, et al., 1997a)</td>
</tr>
<tr>
<td>Zp3-Cre</td>
<td>Female germ line</td>
<td>(Lewandoski, et al., 1997b)</td>
</tr>
<tr>
<td>Protamine-Cre</td>
<td>Male germ line</td>
<td>(O’Gorman, et al., 1997)</td>
</tr>
<tr>
<td>CD19-Cre</td>
<td>B cells</td>
<td>(Rickert, et al., 1997)</td>
</tr>
<tr>
<td>CAG-Cre</td>
<td>Zygotes</td>
<td>(Sakai, et al., 1997)</td>
</tr>
<tr>
<td>tet-Cre</td>
<td>Conditional</td>
<td>(St-Onge, et al., 1996)</td>
</tr>
<tr>
<td>PECAM1-Cre</td>
<td>Early development</td>
<td>(Terry, et al., 1997)</td>
</tr>
<tr>
<td>WAP-Cre</td>
<td>Mammary tissue</td>
<td>(Wagner, 1997)</td>
</tr>
<tr>
<td>MMTV-Cre</td>
<td>Mammary tissue</td>
<td>(Wagner, 1997)</td>
</tr>
</tbody>
</table>

Viral delivery systems for the Cre gene have been developed as an alternative to using transgenic mice. Targeted DNA recombination in vivo has been performed using an adenovirus carrying the Cre recombinase gene (Anton and Graham, 1995; Kanegae et al., 1995; Sakai et al., 1995; Wang et al., 1995;
Kanegae et al., 1996; Akagi et al., 1997; Lee et al., 1997). All cells in which the viral Cre protein is expressed will delete the sequences between the two LoxP sites at both alleles thus creating a tissue specific knockout. This method, however, often produces a mosaic expression pattern for Cre within cells of a given tissue or organ.

1.8: Thesis Objectives

This thesis revolves around the genetic analysis of protein tyrosine phosphatase-epsilon. This PTP exists as two isoforms - a transmembrane-containing receptor-like PTP having a small extracellular domain linked to two tandem phosphatase domains via a transmembrane segment, and an intracellular form containing a unique N-terminus attached to the two tandem phosphatase domains. These isoforms have non-overlapping tissue expression patterns. Upregulation of PTP-ε mRNA in various models of cell differentiation suggests that PTP-ε may have an important role in development of various tissues.

The objectives of this thesis are to determine the functional role of the isoforms of protein tyrosine phosphatase epsilon (PTP-ε) through loss-of-function analysis in the mouse, and to test the hypothesis that murine PTP-ε is required for embryonic development and to determine if the gene product is required for reproduction. A further aim is to examine redundancy issues between the highly related protein tyrosine phosphatases, PTP-ε and RPTP-α.
Chapter 2: Production of Protein Tyrosine Phosphatase Epsilon Mutant Mice

2.1: Introduction

With the aim of studying the developmental and physiological role of PTP-ε we have generated mice mutant for the Ptpre gene. This mutation has been designated PtpreΔ. This chapter describes the experimental approach to the production of PtpreΔ/PtpreΔ mutant mice. This involves the isolation of genomic DNA from the Ptpre locus, its characterization and manipulation to create a pFlox-based conditional targeting vector. The targeting vector was transfected into embryonic stem cells and homologous recombinant clones were identified. Transient transfection of the Cre recombinase permitted the identification of type I and type II deletion clones. These clones were then injected into blastocysts that were then implanted into a host female to produce chimeric offspring. Breeding of the chimeric mice permitted transmission of the mutant allele.
2.2: Results

2.2.1: Partial Cloning of Mouse PTP-ε Locus

In order to develop a targeting vector, a highly specific DNA probe is required for isolation of genomic sequences by screening a genomic library. Typically, a cDNA, labeled to high specific activity, is used as a probe. However, if the cDNA encompasses a very large gene and is highly related to other genes or pseudogenes, this approach may lead to difficulties in characterizing the purified clones. An alternate approach is to develop a specific probe by PCR amplification of intronic sequences. A prerequisite to this approach is the need for some understanding of the intron/exon structure of the gene.

As previously mentioned, Ptpre and Ptpra are highly related at both the nucleic acid and the amino acid levels. Ptpre intron/exon boundaries were predicted based on the genomic structure of mouse Ptpra (Wong et al., 1993). Using Ptpra as a conceptual template, PCR oligonucleotide primers specific for Ptpre were designed (see Materials & Methods for primer sequences) to flank the intron between the two predicted juxtamembrane exons, designated exons 5 and 6 in mouse Ptpre (Wong et al., 1993). Sufficient spacing between the 3' end of the oligonucleotide primers and the predicted splice donor site, in the case of the sense primer, and the predicted splice acceptor site, for the antisense primer, was used to allow unambiguous identification of non-primer Ptpre sequences.
Figure 2.1 A 1.7kb genomic fragment was amplified from 129Sv genomic DNA using primers specific to *Ptpre* but based on the intron/exon structure of the juxtamembrane region of *Ptpra* (see Materials & Methods for PCR conditions).

Using these oligonucleotide primers, a single 1.7kb fragment was amplified from 129Sv genomic DNA (Figure 2.1), subcloned into pBluescript and the ends were sequenced on an automated ABI sequencing instrument. The sequence analysis indicated that the PCR amplified product was derived from PTP-ε genomic sequence and that the intron/exon boundaries were positioned identically to those in *Ptpra* (Wong *et al.*, 1993). The intron/exon boundaries of this region of *Ptpre* are shown in Figure 2.2. The *Ptpre*-specific PCR insert was isolated from the plasmid and the fragment labeled to high specific activity to create a radiolabeled probe. The probe was used to screen a 129SvJ genomic lambda library (Stratagene). Two overlapping clones (18.4 kb and 17.6 kb) were isolated from 1 million plaques screened.
2.2.2: Characterization of the Genomic Clones

Ptpr DNA was isolated from the phage clones and was characterized by restriction mapping and partial sequencing of sub-fragments. These clones were shown to contain the exon corresponding to the transmembrane domain as well as the following two exons containing the juxtamembrane region of Ptpr (Figure 2.2).

![Diagram of the genomic structure showing positions of the three exons (vertical rectangles) and selected restriction enzyme sites. B) The transmembrane domain exon with transmembrane codons underlined. C and D) The two juxtamembrane region exons. Splice donor and acceptor sites are underlined.](image-url)

Figure 2.2: Genomic structure of the transmembrane region of Ptpr. A) Diagram of the genomic structure showing positions of the three exons (vertical rectangles) and selected restriction enzyme sites. B) The transmembrane domain exon with transmembrane codons underlined. C and D) The two juxtamembrane region exons. Splice donor and acceptor sites are underlined.
2.2.3: Vector Design Based on Cre/LoxP System

Given the relative lack of information concerning the developmental expression profile of PTP-ε and its functional role, if any, during development, it was decided to employ the Cre/LoxP technology for the production of Ptpre mutant mice. This would enable the creation of mice having systemic type I deletions (PtpreΔ/PtpreΔ). In addition we also aimed to produce mice containing a floxed PtpreΔ locus (type II deletions), enabling the bypass of any potential for embryonic lethality.

The pFlox vector (kindly provided by Dr. J. Marth) is composed of three LoxP sites flanked by distinct polycloning sites used for cloning of genomic sequences (Hennet et al., 1995). Situated between two of the LoxP sites are the neomycin phosphotransferase positive selectable marker and the thymidine kinase negative selectable marker. Between the adjacent LoxP sites in the pFlox vector is a single BamHI restriction site for cloning of the region to be 'floxed'.

Fragments isolated from the Ptpre specific genomic clones were sequentially subcloned into the pFlox vector creating the targeting vector pFXe2. pFXe2 is composed of a ~1.2kb StuI/EcoRI genomic fragment for the short arm region of homology, a ~1.4kb EcoRI/HindIII genomic fragment encompassing the transmembrane exon and the first cytoplasmic exon as the floxed region, and a ~4.5kb HindIII genomic fragment, containing the second juxtamembrane exon, as the long arm homology region (Figure 2.3). The targeting vector was mapped by using a variety of restriction enzymes to insure that no deletions or rearrangements had taken place during its construction.
Figure 2.3: DNA fragments derived from the endogenous locus were used for constructing the PTP-e targeting vector (for details of construction see section 2.3.3). The targeting vector was electroporated into ES cells and screening was carried out by PCR. Southern blot analysis of clone DNA cut with HindIII using radiolabeled probe A confirmed the identification of homologous recombinant clones. Green arrows indicate LoxP sites, Purple arrows indicate the Tk selection gene, Blue arrows indicate the Neo selection gene, exons are indicated by black rectangles. Red bars show restriction fragments detected by probe A.
2.2.4: Targeting of PTP-ε in ESC by Homologous Recombination

After completion of the targeting vector, the first stage of any targeting project, whether it be a classical targeting project or not, is the introduction of the vector into the embryonic stem cell line and selection for cells carrying the selectable marker. Typically, the introduction is achieved by electroporation and results in one of two possibilities. The most likely event is random integration of the vector into the host genome. At a lower frequency, some cells undergo homologous recombination between the regions of homology in the targeting vector and the corresponding genomic locus. All selection-positive clones must then be characterized as to whether they are homologous recombinants or not. Various screening strategies have been developed over the years to accomplish this.

The linearized pFXe2-targeting vector (30ug) was electroporated into 10 million R1 embryonic stem cells (Nagy et al., 1993) cultured in the presence of LIF. These cells were put under selection with G418 for 14 days. Individual clones were isolated and DNA from each clone was screened by PCR for homologous recombination events between the chromosomal gene loci and the targeting vector. Six independent clones positive by PCR for homologous recombination of 186 clones screened were confirmed by Southern blotting. Digestion of the genomic DNA isolated from PCR positive clones with HindIII resulted in a 3.2kb fragment for the Ptpre<sup>wt</sup> allele when probed with a radiolabeled HindIII/StuI DNA fragment (Probe A in Figure 2.3) and a 2.2kb fragment for the homologously recombined allele when analyzed by Southern blotting (Figure 2.4). Note that the radiolabeled probe does not contain
sequences in common with the targeting vector. This provides further assurances that the *Ptpre* locus is modified in the fashion desired.

The targeting frequency for this gene, that is the ratio of homologously recombined clones to random integration events was approximately 1/30 of all Neo resistant clones.

![Southern blot analysis of genomic DNA isolated from transfected embryonic stem cell clones. DNA was digested with HindIII, transferred to Duralose and probed with Probe A (see Figure 2.3). Lane 1: R1 ESC DNA; Lanes 2-3: ESC DNA from two independent pFlox targeting events. Note that the relative intensities of the wildtype and mutant signals in lane 3 indicate that this clone has parental ES cell contamination.](image)

Figure 2.4: Southern blot analysis of genomic DNA isolated from transfected embryonic stem cell clones. DNA was digested with HindIII, transferred to Duralose and probed with Probe A (see Figure 2.3). Lane 1: R1 ESC DNA; Lanes 2-3: ESC DNA from two independent pFlox targeting events. Note that the relative intensities of the wildtype and mutant signals in lane 3 indicate that this clone has parental ES cell contamination.

2.2.5: Transient Transfection of Cre Recombinase Expressing Plasmid

After determining that the targeting event has taken place in specific ES cell clones and that the homologous recombinant locus' integrity is preserved, subclones are then produced that contain either the type I or type II deletions (Figure 2.5). This is accomplished by introduction by transient transfection of a Cre-recombinase expressing plasmid into the homologous recombinant ES cell
subclones. The Cre-recombinase expressing plasmid, pIC-Cre (kindly provided by Dr. K. Rajewsky), was transiently transfected into one of the homologous recombinant clones. Transfected cells were selected with 1uM gancyclovir, starting 48 hours post-electroporation.

Figure 2.5: Strategy for Cre-mediated recombination in embryonic stem cells of LoxP sites to obtain Type I and Type II deletions. The Cre-expression plasmid was electroporated into recombinant clones and screened initially by PCR. Southern blot analysis of clone DNA cut with XhoI using radiolabeled probe B permitted the identification of clones exhibiting the type I deletion. See Figure 2.3 for description of elements.
A total of 96 colonies were initially assayed by PCR for Cre-mediated deletion of \textit{LoxP} flanked DNA. PCR reactions indicated that all 96 colonies contained type I deletions (data not shown). No type II deletions were observed by PCR. Fourteen clones were picked for further analysis of DNA by Southern blotting. Digestion of the genomic DNA isolated from clones that survived gancyclovir selection following transient transfection of the Cre recombinase into the parental clones was expected to provide, by Southern blot analysis using the XhoI restriction endonuclease and a radiolabeled StuI/EcoRI DNA fragment (Probe B in Figure 2.5), a greater than 12kb band for the endogenous \textit{Ptpre}^{\text{WT}} allele, a 1.3kb band for the type I Cre recombinase-mediated deletion and a 2.7kb signal for the type II Cre recombinase-mediated deletion (Figure 2.5). Digestion of the parental homologously recombined clone DNA was expected to provide a 6.2kb band for the targeted allele when assayed under these conditions.

![Southern blot diagram](image)

**Figure 2.6: Analysis of Cre Transiently transfected ESC DNA by Southern blot.** DNA was cut with XhoI and transferred to Duralose membrane. Probe B (see Figure 2.5) was radiolabeled and hybridized to the membrane bound ESC DNA and washed under stringent conditions. **Lane 1:** Parental homologous recombinant ESC line DNA; **Lane 2 & 3:** Two of fourteen independent type I deletion ESC line DNA. No type II deletions were obtained in this experiment.
2.2.6: Microinjection of Floxed ES Cells into Host Blastocysts

The pFlox targeted ('floxed') embryonic stem cells were microinjected into 3.5 day old blastocysts harvested from C57Bl/6 mice. Since the R1 embryonic stem cells were originally derived from a (129SvJ x 129Sv)F1 mouse they will contribute the agouti coat color. The R1 embryonic stem cell line was derived by Nagy (1993) from the (129Sv x 129SvJ)F1 strain of mice and has an XY constitution. These chimeric blastocysts were then implanted into a pseudopregnant female host. Chimeric progeny derived from these "blastinjects" had varying degrees of agouti and black coat color. The R1 cell line has been shown to contribute to the germline at a high frequency (Nagy et al., 1993). Crossing the strongest chimeric mouse (~95% chimeric) with C57Bl/6 mice resulted in germline transmission of the targeted locus.

2.2.7: pLFpuro: A Novel Strategy for Tissue Specific Ablation

Due to difficulties in obtaining the type II deletion mutant clones, an improved version of the pFlox strategy was developed that involves the use of two recombinase enzymes and their specific target DNA sequences. The use of this system eliminates the random chance of obtaining type I versus type II deletions that occurs with the pFlox strategy as described above.
Paul Orban has kindly provided the pLox² vector containing two LoxP sites separated by a single BamHI cloning site (Orban et al., 1992). Francis Stewart kindly provided the FLP recombinase and a plasmid containing its DNA recognition sequence - the FRT sites (Buchholz et al., 1996). Using standard molecular biology techniques, synthetic oligonucleotides and these components, a novel vector called pLFpuro was created that contains both LoxP sites and FRT sites. The pLFpuro construct consists of two direct repeating FRT sites that flank the PGKpuro positive selectable marker gene and two direct repeating LoxP sites flank this. The PGKpuro cassette contains the puromycin phosphodiesterase positive selectable marker gene driven by the phospho-glycerol kinase promoter. Suitable cloning sites have been introduced to facilitate the construction of targeting vectors with gene-of-interest genomic components. The puromycin resistance gene was chosen as the initial selection method due to its rapid kill rate (Taniguchi et al., 1998). This is important given that ES cells are essentially a primary cell type that with prolonged passaging will undergo differentiation and...
this can lead to the inability of obtaining chimeric mice and/or germline transmission of the mutant allele after blastocyst injection of the modified ES cell clones. Further, the thymidine kinase negative selection cassette was omitted to provide maximum flexibility in the use of this vector given that male mice expressing thymidine kinase are infertile due to disruption of sperm development (Braun et al., 1990). The pLFpuro strategy will allow the production of both type I (total) and type II (floxed) deletions in ES cells as with the pFlox strategy, the difference being the addition of a single FRT site adjacent to one of the LoxP sites in the targeted loci.
CHAPTER 3: Protein Tyrosine Phosphatase-Epsilon Appears Dispensable for Reproduction and Development.

3.1: Introduction

The physiological roles of the two isoforms of PTP-ε are not known. With the aim of studying the developmental and physiological function of PTP-ε mice mutant for the Ptpre gene were generated. Homozygous PtpreΔ/PtpreΔ mutant mice are viable and fertile. PtpreΔ/PtpreΔ mutant mice, when compared to PtpreΔ/Ptprewt and Ptprewt/Ptprewt litter-mate controls, were normal in size and body weight. Further, these PtpreΔ/PtpreΔ mutant mice exhibit no overt behavioral differences from their litter-mate controls. Gross anatomy, X-ray analysis, and histology in these homozygous PtpreΔ/PtpreΔ mutant mice shows no overt difference from wildtype.
3.2: General Analysis

3.2.1: Characterization of Mutant Locus in Mice

Homozygous wildtype \((Ptpre^{wt}/Ptpre^{wt})\), heterozygous \((Ptpre^{Δ}/Ptpre^{wt})\), and mice homozygous for the mutant allele \((Ptpre^{Δ}/Ptpre^{Δ})\) were identified, using restriction fragment length polymorphism analysis of tail DNA, in progeny from a cross of two heterozygous parents (Figure 3.1). This was further confirmed using a PCR based assay developed to distinguish the wildtype allele from the mutant allele (Figure 3.2). This suggests that mutation of the gene for the protein tyrosine phosphatase-epsilon does not result in embryonic lethality and that PTP-ε is not essential for development.

Figure 3.1: Heterozygous and homozygous mutations in the Ptpre allele in mice. Analysis by Southern blotting of tail DNA purified from offspring of heterozygous \((Ptpre^{Δ}/Ptpre^{wt})\) parents. Isolated genomic DNA was digested with MfeI, fractionated through agarose and transferred to Duralose. A radiolabeled probe was hybridized to the immobilized DNA.
The PCR genotyping strategy involves two independent PCR amplification reactions for each sample of tail DNA. The antisense oligonucleotide primer is common to the two PCR reactions for the wildtype and mutant alleles. It anneals to an intronic region falling between the juxtamembrane exons.

The wildtype sense oligonucleotide primer hybridizes to the first juxtamembrane exon and, as such, will not provide a PCR amplification product from \( Ptpre^\Delta/Ptpre^\Delta \) mouse tail DNA when used with the common antisense oligonucleotide primer. The expected wildtype PCR amplification product derived from these two primers is 779bp in length (Figure 3.2).

The mutant allele oligonucleotide primer, when used with the common antisense oligonucleotide primer, amplifies a 508bp PCR product from tail DNA isolated from heterozygotes or \( Ptpre^\Delta/Ptpre^\Delta \) mutant mice (Figure 3.2). The mutant allele sense primer binds to a region 5' of the transmembrane exon. This primer-binding site is common to both the wildtype allele and mutant allele and as such, under ideal conditions can amplify both the 508bp mutant allele PCR product and a >1.5kb wildtype PCR product. However, under the conditions used for rapid genotyping with high throughput, only the mutant allele is amplified from \( Ptpre^\Delta/Ptpre^{\text{wt}} \) tail DNA.
Figure 3.2: Genotyping mutant and wildtype Ptpre alleles by PCR. A. PCR strategy for distinguishing the Ptpre\(^{\Delta}\) allele from the Ptpre\(^{wt}\) allele. Oligonucleotide primers are represented by small arrows and designated S1 and S2 for sense primers, and AS for the antisense primer. B. Analysis by PCR of tail DNA purified from offspring of heterozygous (Ptpre\(^{\Delta}\)/Ptpre\(^{wt}\)) parents. Two independent PCR reactions are required to genotype one sample. The wildtype allele is amplified as a 779bp product using primers S2 and AS1 (upper gel image) whereas the Ptpre\(^{\Delta}\) allele is amplified as a 508bp product using primers S2 and AS (lower gel image). Ptpre\(^{\Delta}\)/Ptpre\(^{\Delta}\) mutant mice in lane 3; Ptpre\(^{\Delta}\)/Ptpre\(^{wt}\) heterozygotes in lanes 2, 4, and 5; Ptpre\(^{wt}\)/Ptpre\(^{wt}\) wildtype mouse in lanes 1 and 6. Details of the PCR reaction are given in Chapter 6.
3.2.2: The \(Ptpre^\Delta\) Mutation Leads to Loss of Cytoplasmic PTP-\(\varepsilon\) mRNA

Analysis of the cytoplasmic transcript of \(Ptpre\) by reverse transcription PCR of total RNA isolated from spleen, thymus and lymph node indicated the loss of the cytoplasmic transcript in homozygous \(Ptpre^\Delta/Ptpre^\Delta\) mutant mice compared to \(Ptpre^{wt}/Ptpre^{wt}\) litter-mate controls (Figure 3.3). Cytoplasmic PTP-\(\varepsilon\) was amplified from the reverse transcribed cDNA library using \(Ptpre\) specific primers that flank the exons deleted in the mutant indicating that deletion of the exons resulted in instability of the PTP-\(\varepsilon\) mRNA. Amplification of mouse \(\beta\)-actin transcript from the reverse transcribed cDNAs indicates that RNA isolation and reverse transcription was successful for all tissues analyzed.

Figure 3.3: RT/PCR from total RNA isolated from spleen, thymus and lymph node indicates loss of cytoplasmic transcript in homozygous \(Ptpre^\Delta/Ptpre^\Delta\) mutant mice compared to \(Ptpre^{wt}/Ptpre^{wt}\) littermate controls. PCR of mouse beta-actin from the cDNAs indicates that RNA isolation and reverse transcription was successful. Details of the RT/PCR reaction are given in Chapter 6.
Due to the very low protein levels of PTP-ε in mouse tissues including brain and testis, western blotting and immunoprecipitation experiments could not be accomplished with the available antibodies. Thus, confirmation of loss of the PTP-ε protein expression and activity in tissues from $Ptpre^\Delta/Ptpre^\Delta$ mice could not be accomplished.

3.2.3: Transmission of Mutant $Ptpre^\Delta$ Allele Follows Mendelian Inheritance.

Homozygous $Ptpre^\Delta/Ptpre^\Delta$ mice are viable and apparently normal. Analysis of the genotype of F1 progeny of double $Ptpre^\Delta/Ptpre^{\text{wt}}$ matings was determined by Southern blot analysis and/or PCR genotyping (for example see Figures 3.1 and 3.2). These results indicate that transmission of the $Ptpre^\Delta$ mutant allele follows Mendelian inheritance patterns. Homozygous mutant, heterozygous and homozygous wildtype progeny occurred with the expected ratio (Table 3.1). This indicates that $Ptpre^\Delta/Ptpre^\Delta$ mice are able to undergo embryonic development in an apparently normal fashion.

Table 3.1: Transmission of mutant allele follows Mendelian inheritance.

<table>
<thead>
<tr>
<th></th>
<th>$Ptpre^{\text{wt}}/Ptpre^{\text{wt}}$</th>
<th>$Ptpre^\Delta/Ptpre^{\text{wt}}$</th>
<th>$Ptpre^\Delta/Ptpre^\Delta$</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Ptpre^{\text{wt}}/Ptpre^{\text{wt}}$</td>
<td>43</td>
<td>106</td>
<td>53</td>
<td>n=202</td>
</tr>
<tr>
<td>$Ptpre^\Delta/Ptpre^{\text{wt}}$</td>
<td>21.3%</td>
<td>52.5%</td>
<td>26.2%</td>
<td>100%</td>
</tr>
</tbody>
</table>
3.2.4: \textit{Ptpre}^{\Delta}/\textit{Ptpre}^{\Delta} Mutant Mice Are Viable, Fertile and Exhibit No Abnormal Behavior

\textit{Ptpre}^{\Delta}/\textit{Ptpre}^{\Delta} female mice were found to be fertile when crossed with \textit{Ptpre}^{\Delta}/\textit{Ptpre}^{\Delta} male mice. The same was true when either male or female \textit{Ptpre}^{\Delta}/\textit{Ptpre}^{\Delta} mutant mice were crossed with stock inbred C57B1/6 mice. \textit{Ptpre}^{\Delta}/\textit{Ptpre}^{\Delta} female mice reared their litters with no apparent difficulties.

All mutant mice exhibited no apparent deviations from normal with respect to feeding behavior, motor activity, circling behavior, and startle responses. No ataxia or tremors were observed in these mice.
3.2.5: Gross Anatomical Review and X-Ray Analysis

Adult $Ptpre^\Delta/Ptpre^\Delta$ mutant mice showed no structural defects in their body plan when compared to $Ptpre^{wt}/Ptpre^{wt}$ mice. Dissection of mutant and wildtype mice indicated no abnormalities in organogenesis. All organs were present in the mutant mice and appeared normal at the gross macroscopic level and at the level of the light microscope.

Development of the skeletal system is a complex phenomenon that involves the growth and remodeling by formation and resorption of bone. Osteoblasts synthesize bone matrix before it calcifies and osteoclasts degrade bone. Due to the presence of PTP-ε transcripts in osteoclasts and osteoblasts (Schmidt et al., 1996), radiographs of $Ptpre^\Delta/Ptpre^\Delta$ mutant mice and $Ptpre^{wt}/Ptpre^{wt}$ mice were taken (Figure 3.4). These results indicated no structural abnormalities in bone development occurred as a result of deletion of the two $Ptpre$ exons from the mouse, nor was there evidence of ostopenia, the generalized reduction in bone mass, or osteopetrosis, the generalized accumulation of bone mass indicative of a defect in bone resorption.

Figure 3.4: Radiographs of $Ptpre^\Delta/Ptpre^\Delta$ mutant mice and $Ptpre^{wt}/Ptpre^{wt}$ mice. (over page)
3.2.6: No Overt Histopathology in Tissue Sections

Changes in histology as a result of gene disruption provides an important clue to the function of many genes. Histological examination under the light microscope of tissues known to express PTP-ε in the wildtype context indicated no obvious difference between mutant (Figures 3.5 to 3.9) and wildtype (data not shown) mice. Tissues in which the wildtype PTP-ε expression status is not known were also examined. All histology sections, obtained from three PtprεΔ/PtprεΔ mice and one wildtype littermate control mouse, were re-examined by Dr. R.D. Cardiff (an expert in mouse histopathology at UCSD) who confirmed the initial observations that no histopathology was evident.
Figure 3.5: Histology of sections through various immune system tissues of $Ptpre^\Delta / Ptpre^\Delta$ mutant mice. a) Thymus (H&E, 10x), b) Thymus (H&E, 100x), c) Lymph node (H&E, 10x), d) Lymph node (Masson’s Trichrome, 100x) e) Spleen showing germinal centers (H&E, 10x), f) Spleen (Masson’s Trichrome, 100x).
Figure 3.6: Histology of sections through various bone and cartilage tissues of
$Ptpre^\Delta/Ptpre^\Delta$ mutant mice. a) (Masson's Trichrome, 10x), b) (Masson's
Trichrome, 10x), c) (H&E, 10x), d) (Masson's Trichrome, 100x), e) (Masson's
Trichrome, 100x), f) (Masson's Trichrome, 100x), g) (Masson's Trichrome, 100x).
Figure 3.7: Histology of sections of $Ptpre^\Delta/Ptpre^\Delta$ mutant mice through the sternum indicating bone and cartilage a) sternum (Masson's Trichrome, 10x), b) sternum (H&E, 10x), c) sternum (H&E, 100x), d) sternum (H&E, 100x)
Figure 3.8: Histology of sections through various tissues of 
$Ptpre^\Delta/Ptpre^\Delta$ mutant mice a) lung (Masson's Trichrome, 10x), b) lung (Masson's Trichrome, 100x), c) heart (Masson's Trichrome, 10x), d) sternum (H&E, 10x), e) salivary glands (Masson's Trichrome, 10x), eye (Masson's Trichrome, 10x).
Figure 3.9: Histology of sections through various gastrointestinal tract tissues of $Ptpre^\Delta/Ptpre^\Delta$ mutant mice. a) Stomach (H&E, 10x), b) Large intestine (H&E, 10x), c) Small intestine (H&E, 10x), d) Bladder (Masson's Trichrome, 10x).
3.2.7: Blood Serum Chemistry

Altered blood chemistry can suggest pathology in organisms. To investigate whether loss of PTP-ε resulted in modified blood chemistry, we undertook blood analysis on eight week old male \( Ptpre^{wt}/Ptpre^{wt} \) and \( Ptpre^{Δ}/Ptpre^{Δ} \) mutant mice from the same litter. Results indicated no major differences in blood chemistry between \( Ptpre^{Δ}/Ptpre^{Δ} \) mutant mice and their littermate controls (Table 3.2).

Table 3.2: Blood chemistry shows no significant difference between \( Ptpre^{wt}/Ptpre^{wt} \) and \( Ptpre^{Δ}/Ptpre^{Δ} \) mutant mice.
3.2.8: Thymocyte Development

Mature αβ T cells arise through a complex process of proliferation and differentiation of multipotent hemopoietic stem cells within the microenvironment of the thymus. T cell development can be monitored by analysis of the expression of the two molecules, CD4 and CD8, on the cell surface of thymocytes. Immature thymocytes are characterized by the lack of CD4 and CD8 expression and are defined as CD4-CD8- or double negative thymocytes. Rearrangement of α and β T cell receptor genes occurs during this stage of development giving rise to double positive (CD4+CD8+) thymocytes. Double positive (CD4+CD8+) thymocytes make up the largest population of thymocytes. The CD4+CD8+ thymocyte population has the potential to represent all possible T cell receptor specificities. Through vigorous positive and negative selection processes, the vast majority of CD4+CD8+ thymocytes die. This selection mechanism generates from the double positive population, mature CD4+CD8- and CD4+CD8+ single positive thymic T cells that exit the thymus to colonize peripheral lymphoid tissues.

Northern blot analysis indicates expression of the cytoplasmic isoform of PTP-ε in the thymus (Elson and Leder, 1995). This suggests a potential role in thymocyte development for this phosphatase. To investigate whether loss of PTP-ε results in modified thymocyte development as measured by CD4/CD8 ratios we undertook FACS analysis of thymocytes of eight week old
*Ptpre*<sup>wt</sup>/Ptpre<sup>wt</sup> and *Ptpre*<sup>Δ</sup>/Ptpre<sup>Δ</sup> mutant mice. The results indicate no major differences CD4/CD8 ratios in between *Ptpre*<sup>Δ</sup>/Ptpre<sup>Δ</sup> mutant mice (Figure 3.8) and their littermate controls (Figure 3.9).

![FACScan analysis of CD4 and CD8 markers on thymocytes of eight week old *Ptpre*<sup>Δ</sup>/Ptpre<sup>Δ</sup> mutant mice.](image)

<table>
<thead>
<tr>
<th>Quad</th>
<th>Thymocytes</th>
<th><em>Ptpre</em>&lt;sup&gt;wt&lt;/sup&gt;/Ptpre&lt;sup&gt;wt&lt;/sup&gt;</th>
<th><em>Ptpre</em>&lt;sup&gt;Δ&lt;/sup&gt;/Ptpre&lt;sup&gt;Δ&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL</td>
<td>CD4+ CD8-</td>
<td>2.22 ± 0.53</td>
<td>1.30 ± 0.39</td>
</tr>
<tr>
<td>UR</td>
<td>CD4-CD8+</td>
<td>78.69 ± 1.18</td>
<td>83.46 ± 3.76</td>
</tr>
<tr>
<td>LL</td>
<td>CD4-CD8-</td>
<td>13.96 ± 0.47</td>
<td>8.82 ± 3.77</td>
</tr>
<tr>
<td>LR</td>
<td>CD4-CD8+</td>
<td>5.13 ± 1.12</td>
<td>6.41 ± 0.40</td>
</tr>
</tbody>
</table>

n = 2  n = 3

Figure 3.10: FACScan analysis of CD4 and CD8 markers on thymocytes of eight week old *Ptpre*<sup>Δ</sup>/Ptpre<sup>Δ</sup> mutant mice.
Chapter 4: Production and Preliminary Characterization of Double Homozygous (PtpraΔ/PtpraΔ; PtpreΔ/PtpreΔ) Mutant Mice

4.1: Introduction

A collaboration has been established with Dr. Jan Sap (New York University Medical Center, Dept. of Pharmacology) to mate mice derived from his Ptpra mutant embryonic stem cells with the Ptpre mutant mice to obtain double mutant mice. The PtpraΔ mutant allele was produced by disruption of extracellular domain exon 3 of the Ptpra gene with an IRES-beta-Geo insertion (Figure 4.1). The IRES-beta-Geo strategy has the added advantage of permitting the histological detection of Ptpra gene expression since the β-galactosidase-neomycin phosphotransferase (beta-Geo) fusion protein is transcribed from the Ptpra gene promoter and translated independently from the internal ribosome entry site (IRES) engineered into the construct.
Mating the \textit{Ptpra} mutant mice with the \textit{Ptpre} mutant mice will permit the determination as to whether genetic redundancy plays a role in the function of these two highly related protein tyrosine phosphatases. Further this experiment will provide insight into the functions of these two protein tyrosine phosphatases through these loss of function studies. We have produced chimeric mice by blastocyst injection of the \textit{Ptpra} mutant ES cells. These chimeric mice have passed the mutant allele through the germline to establish a strain of mice mutant for the PTP-α protein.

Preliminary results from Dr. Sap who has independently establish a strain of mice mutant for the PTP-α protein from the same embryonic stem cell line indicate that \textit{Ptpra}/\textit{Ptpra} mice are viable, fertile and show no overt phenotype. The following analysis will not focus on defining the phenotype of the homozygous \textit{Ptpra}/\textit{Ptpra} mutant mice. This is under current investigation in the laboratory of Dr. Sap. Further, this investigation is only at the most preliminary stage; thus a detailed analysis of the double mutant mice is beyond the scope of this thesis.
Figure 4.1: Targeting strategy uses IRES-beta-Geo to knockout Ptpra. This figure is courtesy of Dr. Jan Sap, NYU.
4.2: Preliminary Results

4.2.1: Characterization of the Ptpra Mutant Locus

As for PCR genotyping of the wildtype and mutant Ptpra alleles, three oligonucleotide primers were used to genotype the wildtype and mutant Ptpra alleles. Fortunately, PCR amplification of both Ptpra alleles can be carried out in a single reaction for each tail DNA sample being tested. The antisense oligonucleotide primer participates in both wildtype and mutant amplification reactions. The sense and antisense oligonucleotide primers used to detect the Ptpra<sup>wt</sup> allele both bind to exon 3 of the mouse gene and produce a 212bp PCR amplification product (Figure 4.2). Detection of the mutant Ptpra<sup>Δ</sup> allele involves the use of a Neo specific sense primer and the common exon 3 antisense primer. Use of these two primers produces a 600bp PCR amplification product (Figure 4.2).

![Figure 4.2: PCR genotyping of the wildtype Ptpra<sup>wt</sup> allele and the mutant Ptpra<sup>Δ</sup> allele. Each lane represents the PCR product amplified from tail DNA isolated from an individual mouse.](image-url)
Combining the \textit{Ptpra} PCR genotyping strategy with the \textit{Ptpre} PCR genotyping strategy describe in Chapter 3, we can trace all four alleles in tail DNA from progeny of crosses between these two strains of mice. The first breeding goal is to identify double heterozygotes ($\text{Ptpra}^\Delta/\text{Ptpra}^\text{wt}; \text{Ptpre}^\Delta/\text{Ptpre}^\text{wt}$) from crosses between the two strains. Once identified, crossing of double heterozygous male and female mice is expected to lead to creation of double homozygous ($\text{Ptpra}^\Delta/\text{Ptpra}^\Delta; \text{Ptpre}^\Delta/\text{Ptpre}^\Delta$) mutant mice at an expected frequency of $1/16$ of all progeny according to Mendel's rules.
Chapter 5: Discussion

With the aim of studying the developmental and physiological role of PTP-ε we have generated mice mutant for the Ptpre gene. This mutation has been designated PtpreΔ. The initial step in creating PtpreΔ mice was the isolation and characterization of locus specific DNA from a 129SvJ mouse genomic library using a PCR-generated radiolabeled probe. Characterization of the genomic DNA indicated conservation of the intron/exon boundaries between the transmembrane exon and the two exons corresponding to the juxtamembrane region of the protein encoded by the murine Ptpre gene and the murine Ptpra gene (Wong et al., 1993). The intronic regions flanking this cluster of exons were, as expected, not conserved. It is predicted that the remainder of the genomic structure for Ptpre with the exception of the extracellular region and the 5' exon(s) of the cytoplasmic isoform will be highly similar to that of Ptpra. Further characterization of the genomic structure of Ptpre will be required to confirm this. This would also establish whether distinct promoters regulate the expression of the two isoforms of PTP-ε.
The juxtamembrane region of the receptor-like form is also found in the intracellular form and is defined by two exons in the mouse genome. Through deletion of the transmembrane and first juxtamembrane exons of the Ptpre gene, equivalent to exons 4 and 5 in the Ptpra gene, in mouse R1 embryonic stem cells by homologous recombination, followed by Cre-mediated excision of LoxP flanked exons, we have generated mice mutant for these two forms of PTP-e.

Homozygous PtpreΔ/PtpreΔ mice were viable and fertile. Gross anatomy in these homozygous mutant mice revealed no overt difference from wildtype. PtpreΔ/PtpreΔ mice, when compared to PtpreΔ/Ptprewt and Ptprewt/Ptprewt littermate controls, are normal in size and body weight (data not shown). PtpreΔ/PtpreΔ mice exhibited no overt behavioral differences from their littermate controls. Radiographic analysis, and histology of PtpreΔ/PtpreΔ mice revealed no differences from wildtype. No significant differences in blood chemistry parameters were observed between PtpreΔ/PtpreΔ mice and their littermate controls. FACS analysis of CD4/CD8 developmental profiles of thymocytes showed no significant difference between PtpreΔ/PtpreΔ mice and Ptprewt/Ptprewt littermate controls.

These results are in agreement with the unpublished work of Ari Elson who created Ptpre null mice using the classical gene targeting strategy by replacing the exons encompassing the first phosphatase domain of PTP-e with a selectable marker (Ari Elson, personal communication). No novel phenotype has
been observed in his PtpreΔ/PtpreΔ colony to date, despite intensive investigation.

Protein tyrosine phosphatase-epsilon shows the strongest amino acid similarity to the receptor-like PTP-α (Figure 1.7). Given the high degree of similarity between these two tyrosine phosphatases it is reasonable to hypothesize that these molecules may, at least in some tissues, be functionally redundant. In collaboration with Dr. Jan Sap we have crossed PtpraΔ/PtpraΔ mice with PtpreΔ/PtpreΔ mice to create a double "knockout" line. These matings resulted in viable PtpraΔ/PtpraΔ:PtpreΔ/PtpreΔ offspring that upon brother-sister mating produced viable progeny indicative of normal fertility, lactation and offspring care. Further detailed analysis is required to ascertain if a subtle phenotype is present but not uncovered in the analysis performed to date or if another, known or unknown, protein tyrosine phosphatase is compensating for the loss of these two molecules.

There are many examples of gene knockout experiments in the literature that result in either no phenotype or a much milder phenotype than predicted. For example, deletion of the extracellular matrix protein, tenascin, resulted in mice with no observable phenotype (Saga et al., 1992). This led the authors to cast doubt on the hypothesis that tenascin had an essential role in development as predicted from in vitro and in vivo studies.
The cytoskeletal phosphoprotein tensin is expressed at focal adhesion in many different tissues during mouse development and associates with F-actin (Guan, 1997). Mice having a disruption of the tensin gene unexpectedly developed normally and appeared healthy post-natally for at least several months (Lo et al., 1997). Over time, tensin-/− mice became weak due to the development of multiple cysts in the proximal kidney tubules and, as a result, show signs of renal failure. Focal adhesion junctions could be found in noncystic areas of the kidneys of tensin-/− mice. In regions with cysts, focal adhesion junctions were disrupted. These authors found that all other tissues appeared normal. This suggests tensin's functions are redundant and may be compensated for by other focal adhesion proteins.

The accumulation of inactivating mutations is expected to occur in genes that are uncoupled from the forces of natural selection. In spite of this, in the wide variety of organisms studied, ranging from prokaryotic to eukaryotic species, it has become apparent that gene deletions that lead to no obvious phenotype are a common observation (Miklos and Rubin, 1996). A number of hypotheses have been developed to try and explain this paradox. The hypothesis that many genes have necessary functions that are revealed only in response to specific stresses or environments not encountered in the laboratory setting has been termed the "contingent function" hypothesis.

The "contingent function" hypothesis implies that conditional phenotypes will be revealed under the right experimental conditions (Thatcher et al., 1998). Since the immune system is dispensable in a sterile environment such as the
laboratory, many molecules of the immune system can be deleted from the mouse without apparent adverse effect on the animal in this setting. Yet, if these animals are, for example, challenged by a pathogen not normally encountered then a phenotype may become apparent. For example, mice in which the interferon-gamma gene was disrupted are unable to control a normally sublethal dose of M. tuberculosis (Cooper et al., 1993; Flynn et al., 1993). A wide body of knowledge has implicated interferon gamma as a principal mediator of macrophage activation and resistance to intracellular pathogens.

Identification of subtle phenotypes resulting from induced mutations can be difficult, as important function of genes may not reveal themselves without the appropriate stresses or environment. Further, phenotypes may be difficult to observe unless one knows where to look.

Functional redundancy between members of a gene family can obscure a phenotype through loss of function of one of the members. For example, MyoD, a member of the myogenic basic helix-loop-helix transcription family, is thought to regulate along with other members of this family differentiation of skeletal muscle. Creation of homozygous null mutant mice by deletion of the MyoD gene resulted in apparently normal muscle development (Rudnicki et al., 1992). These authors suggest that another member of this family, Myf-5, may compensate for the loss of MyoD and these authors subsequently found that the Myf-5 mRNA was upregulated in the MyoD mutant mice. This suggests that functional redundancy can occur between these two, and perhaps other, myogenic factors from this gene family. However, mice lacking Myf-5 died post-natally due to the
absence of the major distal part of the ribs (Braun et al., 1992). Myf-5, interestingly, was found to be dispensable for skeletal muscle development, perhaps because other members of the myogenic HLH family are able to substitute for Myf-5 activity. Mice lacking both MyoD and Myf-5, although born alive, lacked evidence of muscle activity and died soon after birth (Rudnicki et al., 1993). Northern blot and S1 nuclease analyses indicated that Myf-5/MyoD double mutant mice expressed no detectable skeletal muscle-specific mRNAs. These results indicate that functional redundancy does occur in the myogenic basic HLH family of transcription factors.

The Src family protein tyrosine kinases are another example of a family of signaling molecules showing varying degrees of redundancy, as a result of deletion of the individual Src PTKs from the mouse genome (Lowell and Soriano, 1996; Thomas and Brugge, 1997). In the mouse there exist eight members of this family all showing significant amino acid homology to the family prototype, the Src proto-oncogene. The Src PTKs included the broadly expressed molecules Src, Fyn, Yes, and Lyn as well as the hematopoietic-restricted molecules Fgr, Hck, Lck, and Blk. There exists a wide body of literature indicating that the Src PTKs are involved in regulation of a broad range of biological phenomena (Thomas and Brugge, 1997).

Individual deletions of all known mammalian Src PTKs by gene targeting have been made in mice (Lowell and Soriano, 1996). Src mutant mice suffer osteopetrosis due to an intrinsic defect in osteoclasts (Soriano et al., 1991; Boyce et al., 1992; Lowe et al., 1993), whereas thymocyte blockage was observed in lck-/-
mice and fyn-/- mice showed TCR signaling defects in thymocytes (Stein et al., 1992). In contrast, mutations in Yes (Stein et al., 1994), Hck (Lowell et al., 1994), Fgr (Lowell et al., 1994), and Blk (cited in Lowell and Soriano, 1996) showed either subtle or no observable phenotype. Double and triple mutant mice have been generated by mating together the various single mutant Src PTKs mice. These experiments have indicated that functional redundancy by the various members occurs. For example, doubly mutant hck-/-;fgr-/- mice exhibit an increased susceptibility to Listeria monocytogenes infection, a bacterial pathogen (Lowell et al., 1994). No increased susceptibility to this pathogen was observed in either of the single mutant mice.

The "marginal benefit" hypothesis is an alternative, though not mutually exclusive, hypothesis which states that many genes may never be essential but instead make small contributions to the efficiency and/or reliability of systems under ordinary conditions (Thatcher et al., 1998). This implies that a phenotype resulting from a particular gene knockout is present but may not be detectable by methods currently available.

Strain differences, for example, can have a significant impact on the expression of mutant phenotypes. For example, the LAR mutant mice had incomplete penetrance of the observed phenotype (Schaapveld et al., 1997). In spite of LAR's broad expression, these mice developed and grew normally and exhibited no overt histological tissue abnormalities. Mammary glands of LAR mutant females, however, were incapable of delivering milk due to an impaired terminal differentiation of alveoli at late pregnancy. Interestingly, this
phenotype had variable penetrance with the mutation bred on a mixed 126Sv x C57Bl/6 background. However, when the mutation was bred onto the C57Bl/6 strain, penetrance was complete. This suggests the presence of an, as yet, unidentified modifier gene(s) in one strain.

Clearly, further analysis of both PTP-ε and RPTP-α are required in order to understand the functions of these molecules in mammalian cells. With this aim, a collaboration has been established with Dr. Niels Peter H. Møller (Senior Scientist, Novo Nordisk, Denmark) using the Ptpre^ / Ptpre^ mutant mice to study the possible role that PTP-ε plays in dephosphorylating the insulin receptor and glucose metabolism in vivo. Measurement of insulin, insulin receptor kinase activity and glucose tolerance tests will be performed in his lab. Dr. Møller previously showed in baby hamster kidney cells overexpressing the insulin receptor a selective down regulation of the insulin receptor signal by transfection of RPTP-α and PTP-ε but not by LAR, PTP-μ, CD45, PTP-1B, PTP-1D, PTP-1C, PTP-H1 nor PTP-D1 (Moller et al., 1995).

Further analysis of these mice should investigate the expression of other protein tyrosine phosphatases to see if any are upregulated compared to control mice. With regards to the hemopoietic system, a more detailed functional analysis of T cell receptor signaling in thymocytes and mature T cells and of monocyte/macrophage function might shed light on the role of these molecules. For example, infection of these mice with Listeria monocytogenes might indicate an
increased susceptibility to this pathogen thus pointing to a defect in macrophage function.

Further experiments could include expression of dominant-negative mutants (catalytically inactive forms that can form stable complexes with molecules required for the PTPs functions) of PTP-ε using transgenic or gene targeting technology. Through this approach, identification of signal transduction pathways impacted by this protein tyrosine phosphatase may be possible.
Chapter 6: Materials and Methods

6.1: For Chapter 2

6.1.1: Isolation of PTP-epsilon Genomic Sequences

PTP-epsilon intron/exon boundaries were predicted from the genomic structure of mouse RPTP-alpha. Using RPTP-α as a template, PCR oligonucleotide primers specific for PTP-ε were designed to flank the intron between the two predicted juxtamembrane exons, designated exons 5 and 6 in mouse RPTP-α.

Isogenic genomic DNA was isolated from 129/SvJ-mouse kidney to use as a template to amplify a PTP-ε specific probe by PCR. The primers SIC-1 (CGC GGA TCC AAC GAC AAG AAA ATG CCT AAC GGG) and ASIC-2 (CGC GGA TCC TGG AGA TCT GCT CAG CAG CAT CAC) were used in the PCR reaction to isolate the probe DNA. PCR conditions used (95°C, 45 seconds; 55°C, 1
minute; 72°C, 1 minute 30 seconds + 2 seconds/cycle) for 35 cycles. A ~1.7kb PCR product was gel purified after fractionation on a 1% agarose/TAE gel using the geneclean isolation procedure. The ends of the PCR fragment were digested with BamHI to facilitate subcloning into pBluescript thus creating the plasmid pBS129. Sequencing of the insert ends with M13 forward and reverse primers indicated that the PCR product was derived from the mouse PTP-ε locus.

The PCR genomic fragment was isolated from the plasmid and labeled with $^{32}$Pα-dCTP by random priming with Klenow DNA Polymerase. This radioactive probe was purified from unincorporated radionucleotide, denatured and used to screen a 129SvJ genomic liver DNA library in lambda FIX II (Stratagene). Hybridization of the probe to the plaque filters was performed in Hybridization Buffer at 42°C overnight in roller bottles. After hybridization the filters were rinsed once at RT and then washed twice at 55°C in 0.1xSSC, 0.1%SDS for 15 minutes. The filters were exposed to Kodak XAR-5 autoradiography film for 24 hours. Two positive signals were detected from one million plaques screened and purified to homogeneity by successive rounds of hybridization with the radiolabeled PTP-ε specific genomic probe.

These two PTP-ε positive phage clones were expanded in liquid culture and purified using the CsCl₂ method. Phage DNA was isolated from the clones and subjected to restriction digest with a variety of restriction endonucleases. The digests were separated on a 0.7% agarose/TAE gel, photographed, denatured and then transferred to Duralose membrane for Southern blot analysis with the pPCR129 derived radiolabeled probe. Southern blot analysis indicated that the lambda clones were overlapping but independent clones. These clones
were shown to contain the exon corresponding to the transmembrane domain as well as the following two exons containing the juxtamembrane region of PTP-ε.

Restriction fragments that were positive for the probe in the Southern blot analysis, were isolated and subcloned into pBluescript for further analysis. Digestion of the phage clone DNA with NotI liberated the genomic fragment thus facilitating its subcloning into pBluescript creating the plasmid pBS224. Restriction digest analysis and sequencing of the various subfragments and pBS224 resulted in the identification of three exons having 100% nucleotide identity with the transmembrane region of mouse PTP-ε.

6.1.2: Targeting Vector Construction

Three fragments derived from the PTP-ε genomic DNA were sequentially cloned into the pFlox vector to create the final targeting vector, pFXe2. Figure 2.3 indicates the genomic fragments used, and their relative positions, in the construction of pFXe2. First, the ~4.5kb HindIII fragment from pBSH34.5 was cloned into the HindIII site of the pFlox vector to provide the long arm for homologous recombination. The correct orientation of the long arm was determined by restriction digest.

To facilitate the cloning of the floxed region into the BamHI site of the pFlox vector a new general-purpose pBluescript derivative was constructed by the introduction of a BamHI linker (CGGATC) into the ClaI site of pBluescript. This vector, pBBBS, maintains the blue/white selection capabilities of the parental vector. The HindIII/EcoRI fragment containing exons 4 and 5 was
cloned directionally into pBBBS creating pBBBSe4&e5. Subsequently the 450bp HindIII fragment from pPCR129 was inserted into the HindIII site of pBBBSe4&e5 and screened for orientation with SacI creating pBBBSe4&e5H3. The floxed region was liberated from pBBBSe4&e5H3 by digestion with BamHI and cloned into the unique BamHI site of pFX4.5. Screening for correct orientation created pFXB.

The ~1.2kb StuI/EcoRI fragment isolated from pBSH33.0 was cloned into the Smal/EcoRI site of pSSBS. pSSBS is a general-purpose vector based on pBluescript in which the XbaI site was converted to a SacI site in such a way as to maintain blue/white selection. The ~1.2kb short arm was liberated with SacI and cloned in the correct orientation into the unique SacI site of pFXB to create the completed targeting vector, pFXe2. The targeting vector was mapped by using a variety of restriction enzymes to insure that no deletions had taken place during its construction.

6.1.3: Targeting of PTP-epsilon Locus in Embryonic Stem Cells

All embryonic stem cell culturing was performed using R1 media consisting of DMEM supplemented with non-essential amino acids, sodium pyruvate, L-glutamine, Penicillin/Streptomycin, β-mercaptoethanol and 10% fetal calf serum. The fetal calf serum was lot tested with R1 embryonic stem cells to ensure that it maintained the cells in a pluripotent state.
The NotI-linearized targeting vector (30ug) was electroporated into 10 million R1 embryonic stem cells (ESC) cultured in the presence of LIF. These cells were put under selection with 180ug/ml G418. Individual clones were picked into TE. One half of the clone colony was transferred onto gelatinized 24 well tissue culture plates and the other half was used to isolate DNA for PCR analysis. DNA from each clone was pooled into lots of eight and screened by PCR to differentiate between homologous recombination events between the chromosomal gene loci and the targeting vector and random integration of the vector into the genome. Six independent clones positive by PCR for homologous recombination were confirmed by Southern blotting.

6.1.4: DNA Isolation from ESC Clones

Trypsinized ES cells were washed twice in ice cold PBS. Pelleted cells were re-suspended in 100μg/ml Proteinase K, 0.1% SDS, 50mM Tris-HCl pH 8.0, 50mM EDTA and incubated at 55°C for 1-3 hours. DNA was purified after one round of phenol/chloroform extraction, one round of chloroform extraction followed by precipitation of the DNA with an equal volume of isopropanol. The DNA pellet was washed with 70% ethanol and re-suspended in TE.

6.1.5: PCR Screening and Southern Blot Analysis of ESC Clone DNA

PCR screening to distinguish between ES cell clones with random vector integration and those with homologous recombination at the Ptpre locus required
a nested PCR strategy. Two oligonucleotide primers were to the promoter region of the neomycin phosphotransferase gene and as such were specific to the pFlox-targeting vector. These primers were TkB ( ) and Tk42 (GGT CCC TCG ACC TGC AGC CCA AGC TGA TCC). The two antisense Ptpre locus-specific oligonucleotide primers were to an intronic region that did not hybridize to the targeting vector. These were the outer primer P8 (CCT ATG CCT CCC TGG GTG GA) and the inner primer P7 (CCC ACC CAG CTG GCT CTG GGT).

The first PCR reaction used the outer most primer pair and was expected to yield a ~1.4 kb amplification product. PCR conditions for the first PCR reaction were (95°C, 30 seconds; 60°C, 30 seconds; 72°C, 50 seconds) for 30 cycles followed by a soak at 72°C for 10 minutes. The second PCR reaction used the inner most primer pair and yielded a ~1.2 kb amplification product. PCR conditions for the second PCR reaction were (95°C, 30 seconds; 55°C, 30 seconds; 72°C, 40 seconds) for 30 cycles followed by a soak at 72°C for 10 minutes. Amplification products were analyzed on a 1.5% agarose/TAE gel.

6.1.6: Transient Transfection of Cre Recombinase Expressing Plasmid

The Cre recombinase expressing plasmid, pIC-Cre (kindly provided by Dr. K. Rajewsky (Gu et al., 1993)) was transiently transfected into one of the homologous recombinant clones. Transfected cells were selected with 1uM Gancyclovir. Fourteen clones were picked for further analysis by Southern blotting.
6.1.7: Microinjection of Floxed ESCs into Blastocysts to Generate Chimeric Mice

pFlox targeted (Floxed) embryonic stem cells were pre-plated for one hour at 37°C to remove PEFs. During pre-plating, PEFs adheres to the tissue culture plates faster than the ES cell clones thus permitting their removal from the ES cells. Microinjection of 10-15 ES cells into 3.5-day-old blastocysts harvested from C57Bl/6 mice. Microinjected chimeric blastocysts were surgically implanted into the uteri of pseudo-pregnant outbred female mice.

Since the R1 embryonic stem cells were originally derived from a (129SvJ x 129Sv)F1 mouse they will contribute the agouti coat color. The R1 ES cell line was derived by Nagy (1993) from the 129/Sv strain of mice and has an XY constitution. Chimeric progeny derived from these "blastinjects" have varying degrees of agouti and black coat color. Crossing the chimeric mice with C57Bl6 mice results in germline transmission of the targeted locus.

The modified ES cells were microinjected into the host blastocysts. Generally 8-15 ES cells are injected into the blastocoel cavity. These chimeric blastocysts were then implanted into a psuedopregnant female host. Since the ES cells used for generating the gene modifications have a male karyotype, analysis of progeny need only concern inspection of male offspring. The time required for generation of chimeric mice exhibiting ES cell contribution to the germline varies considerable and is partly dependent of which ES cell line is
used. The R1 cell line has been shown to contribute to the germline at a high frequency (Nagy et al., 1993).

6.2: For Chapter 3

6.2.1: Southern Blot Analysis of Tail DNA

Analysis by Southern blotting of tail DNA purified from mice permitted the analysis of genotypes with respect to the Ptpre locus. Approximately 1 cm of tail was surgically removed from methoxyfluorane anaesthetized mice. Tail samples were re-suspended in 100μg/ml Proteinase K, 0.1% SDS, 50mM Tris-HCl pH 8.0, 50mM EDTA and incubated at 55°C overnight. DNA was purified after one round of phenol/chloroform extraction followed by precipitation of the DNA with an equal volume of isopropanol. The DNA pellet was washed with 70% ethanol and re-suspended in TE. Isolated genomic DNA was digested with the appropriate restriction enzyme, fractionated through agarose and transferred to Duralose. A radiolabeled probe was hybridized to the immobilized DNA.

6.2.2: Reverse Transcription of Mouse Tissue RNA

Total RNA was isolated from various tissues of both mutant and wildtype littermates using the Trizol reagent according to manufacturers instructions.
Reverse transcription of this total RNA to cDNA was performed using Superscript Reverse Transcriptase. As a control for quality of the cDNA reaction for each tissue sample, mouse β-actin was amplified by PCR using specific primers (Stratagene). Primers specific to mouse PTP-ε cDNA that flank the region deleted in the mutant mice were designed to test for the presence or absence of the PTP-ε transcript. The sense primers (SIC-1 CGC GGA TCC AAC GAC AAG AAA ATG CCT AAC GGG for the cytoplasmic isoform mRNA and MEx for the receptor-like isoform mRNA) and the antisense primer (ASIC-2 CGC GGA TCC TGG AGA TCT GCT CAG CAG CAT CAC) were used in the PCR reactions to amplify the cDNA. PCR conditions (95°C, 20 seconds; 55°C, 45 seconds; 72°C, 45 seconds + 2 seconds/cycle) used for 35 cycles.

6.2.3: PCR Assay of Tail DNA

Homozygous wildtype (Ptpre<sup>wt</sup>/Ptpre<sup>wt</sup>), heterozygous (Ptpre<sup>Δ</sup>/Ptpre<sup>wt</sup>), and mice homozygous for the mutant allele (Ptpre<sup>Δ</sup>/Ptpre<sup>Δ</sup>) are observed, using restriction fragment length polymorphism analysis of tail DNA, in progeny from a cross of two heterozygous parents (Figure 3.1). This is further confirmed using a PCR based assay developed to rapidly distinguish the wildtype allele from the mutant allele.
6.2.4: Histology

CO2 asphyxiated mice were dissected for various samples of tissue. Tissue samples were collected in tubes containing 4% paraformaldehyde/ phosphate buffered saline and were allowed to fix for 48 hours at room temperature. Bone samples contain calcified areas which was decalcified in 8% formic acid before processing and sectioning. Processing of samples was performed using automated tissue processing equipment. Paraffin embedded tissue samples were sectioned and stained with either haematoxylin and eosin or Massons trichome. For haematoxylin and eosin (H&E) staining, paraffin sections were taken to water and placed in haematoxylin for 5 minutes then rinsed in tap water. Sections were 'blued' in lithium carbonate and washed in tap water. Following this sections were placed in 1% acid alcohol for a few seconds, washed in tap water and placed in eosin for 5 minutes then rinsed. For Massons trichome staining, paraffin sections were taken to water and nuclei were stained with Weigerts iron haematoxylin for 10 minutes. Sections were then washed in water and 'blue' in lithium carbonate and stained in 1% panceau-acetic acid solution for 5 minutes. After a rapid rinse in water, sections were placed in 1% phosphomolybdic acid for approximately 5 minutes, then drained and counterstain with aniline blue.
6.2.5: Radiographic Studies

X-ray studies of the mice were performed using a Hewlett Packard Faxitron at 50 kVP and 34 mA. Animals were anesthetized using methoxyfluorane during radiographic examinations.

6.2.6: Blood Serum Chemistry

Mice were anaesthetized with 0.4 ml of 2.5% Avertin, blood was drawn via a heart puncture. Serum was isolated by centrifugation of blood samples that had been allowed to clot at room temperature for 30 minutes. Serum was analyzed for blood chemistry using a Kodak Blood Analyzer (Vancouver General Hospital).

6.2.7: FACScan Analysis

Thymocytes and lymph node T cells were harvested from mice as single cell suspensions by mincing tissues through wire-mesh screens in ice cold 5% serum in FACS buffer (Becton Dickinson, Mountainview, CA). Cells were incubated with fluorochrome-conjugated antibody (FITC and PE, Becton Dickinson, Mountainview, CA) at a density of 5 x 10^6 cells/ml on ice and then washed in cold FACS buffer. A Becton Dickinson non-sorting FACScan flow cytometer equipped with a Macintosh running CellQuest was used for analysis.
6.3: For Chapter 4

6.3.1: PCR Genotyping of Ptpra Alleles

As for PCR genotyping of the wildtype and mutant Ptpra alleles, three oligonucleotide primers are used to genotype the wildtype and mutant Ptpra alleles. Fortunately, PCR amplification of both Ptpra alleles can be carried out in a single reaction for each tail DNA sample being tested. The antisense oligonucleotide primer participates in both wildtype and mutant amplification reactions. The sense (AES-6 CCT GAC TCT GGA GCC CAC C) and antisense (AE-3 CTG CCG GGG GGA AGGT TTC) oligonucleotide primers used to detect the Ptpra\textsuperscript{wt} allele both bind to exon 3 of the mouse gene and produce a 212bp PCR amplification product. Detection of the mutant Ptpra\textsuperscript{A} allele involves the use of a Neo specific sense primer (Neo1194 GGT GTG GCG GAC CGC TAT C) and the common exon 3 antisense primer (AE-3 CTG CCG GGG GGA AGG TTT C). Use of these two primers produces a 600bp PCR amplification product. PCR conditions are (95°C, 30 seconds; 60°C, 30 seconds; 72°C, 50 seconds) for 30 cycles followed by a soak at 72°C for 10 minutes.
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