The Cloning and ~~Preliminary~~ Characterisation of Limk1: a LIM-Domain Containing Protein Kinase

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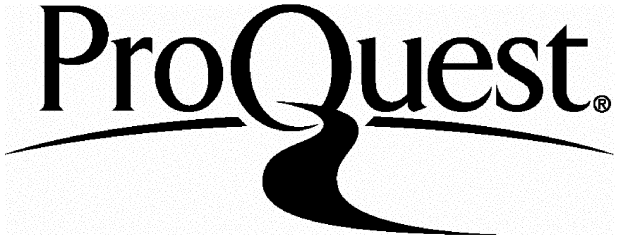

zerbrechlich
zeitlos
vielfältig - faszinierend
unergründlich

die schöpfung - leben
und alles
was zum werden
bestimmt ist.

durch Margot und für Sophia
london, zum 1. märz 1995

Abstract

This thesis describes the isolation and cloning of a novel mouse gene, named *mLimk1*, which exhibits high homology to the human LIMK gene. *mLimk1* represents a single copy gene and maps to the distal end of mouse chromosome 5. Northern blot analysis showed preferential expression of a 3.5kb message in adult spinal cord and brain. *In situ* hybridisation studies confirmed high expression levels in the nervous system, particularly in the spinal cord and the cranial nerves and dorsal root ganglia. The amino acid sequence reveals two features which place mLimk1 into a novel class of protein kinases. Firstly, although mLimk1 contains all motifs found in catalytic kinase domains, amino acids previously described to be diagnostic of either serine/threonine- or tyrosine-kinases are not present. It is demonstrated that mLimk1-fusion protein can autophosphorylate on serine, tyrosine and threonine residues *in vitro*, and mutation of residue D460 within the IHRDL motif abolishes kinase activity. Secondly, mLimk1 has two tandem LIM-domains in the amino-terminal region. These zinc-finger like domains can mediate protein-protein interactions and have been described in transcription factors and cytoskeletal proteins. The combination of LIM- and kinase domains may provide a novel route by which intracellular signaling can be integrated.

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List of Abbreviations

Accepted abbreviations in this thesis are listed in Biochem. J. (1992) 281, p.11–12. In addition the following abbreviations are used:

ALL	Acute lymphocytic leukemia
ATP	Adenosine tri-phosphate (Mg salt)
β MeOH	β Mercaptoethanol
BSA	Bovine serum albumin
cAMP	Cyclic adenosine mono-phosphate
cAPK	cAMP-dependent protein kinase, catalytic subunit
CML	Chronic myelogenous leukemia
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
E(n)	Day n of embryonic development
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAP	GTPase activating protein
GST	Glutathione S-transferase
GSH	Reduced form of the glutathione tripeptide
HD	Homeodomain of LIM-homeodomain proteins
HGF	Hepatocyte growth factor
IFN	Interferon
IgG	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IPTG	Isopropyl-2-D-thiogalactopyranoside
ISH	*In situ* hybridisation
KD	Catalytic kinase domain of protein kinases
MAP kinase	Mitogen-activated protein kinase
MEK/MAPKK	Activating kinase of MAP kinase
NP-40	NonidetP-40
NMR	Nuclear magnetic resonance spectroscopy
NTP	Nucleotide tri-phosphate

O-2A	Oligodendrocyte-type 2 astrocyte
p.c.	Post coitum
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulphonyl fluoride
PTK	Protein tyrosine kinases
Prl	Prolactin
PSK	Protein serine/threonine kinases
PVDF	Polyvinylidene difluoride
RACKs	Receptors for activated C-kinase
RTK	Receptor tyrosine kinases
RT-PCR	Reverse transcriprase rewaction followed by PCR
Sf9	Spodoptera frugiperda-9
SH2/3 domain	Src homology 2/3 domain
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40
TLC	Thin layer chromatography
TSHβ	Thyroid-stimulating hormone β
Tween 20	Polyoxyethylenesorbitan monolaurate

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Introduction

1.1. General introduction

In the last few years tremendous progress has been made towards understanding how extracellular signals are transmitted by cascades of signalling molecules from the cell surface to the nucleus, where they can bring about changes in gene expression. The outcome of such signalling depends much on the cellular context in which the signal is transmitted. And yet, despite the large number of players involved in this process and the high degree of cross-talk between parallel signalling pathways, the transduction and integration of signals remains a tightly regulated and surprisingly specific process. This specificity is not only determined by the substrate specificity of each of the signal molecules involved, but can also be enhanced by the formation of multiprotein complexes, by attenuating mechanisms which inactivate an incoming signal and by the requirement for combinatorial interactions between transcription factors (reviewed in Hill and Treisman 1995). Moreover, it has also become clear that the response of a cell to a particular signalling molecule depends not only on the *qualitative* differences between the signal transducing molecules but also on the *quantitative* differences between signals (Traverse *et al.* 1992; Marshall 1995). In other words not only the nature of the signalling molecules involved but also the intensity of a signal will determine the outcome of an inducing signal. These threshold mechanisms enhance signal specifity and allow the integration of different incoming signals to modulate the resulting cellular response.

The context in which a signal is processed by a cell is determined by the molecules present in the cell. This in turn is a function of the developmental history of the cell. Thus different cell-types often provide a different context to a single signalling molecule. In order to understand the processes occuring in different cell types, it will eventually be necessary to identify all players in this increasingly complex network of signal and effector molecules. This will require an enormous and concerted effort of the scientific community. The work presented here describes the cloning and preliminary characterisation of one new signalling molecule, a protein kinase.

1.2. Protein kinases: key players in signal transduction

1.2.1 To ~Ⓟ or not to ~Ⓟ: protein phosphorylation is here to stay

In 1955 Krebs and Fisher discovered that glycogen phosphorylase is activated through the reversible addition of an activated phosphate group, ~Ⓟ (Fisher and Krebs 1955). Such protein phosphorylation can alter the equilibrium between active and inactive states of an enzyme by inducing allosteric conformational changes (Sprang *et al.* 1988) as well as by electrostatic effects (Hurley *et al.* 1990). We now recognise that protein phosphorylation is a fundamental mechanism in regulating numerous cellular processes, including signal transduction. The enzymes catalysing the transfer of the γ-phosphate group of MgATP to a protein substrate are known as protein kinases (Hunter 1991).

1.2.2 Protein phosphorylation is a major currency of signal transduction

Protein kinases are integral components of several signalling pathways, and can modulate the interactions of signalling molecules in essentially two ways. First, by altering the enzymatic activity of downstream proteins. An example of such an interaction is the activation of insulin receptor kinase activity following tyrosine phosphorylation of the β subunit (Yu and Czech 1984). Conversely, phosphorylation of threonine^{654} of the epidermal growth factor receptor inhibits its kinase activity (Countaway *et al.* 1990). Another mechanism by which kinases regulate signalling pathways is by controlling the formation of complexes between signalling and effector molecules. This is exemplified by the formation of high affinity binding sites for SH2-domain containing molecules through tyrosine phosphorylation of SH2 binding sites (reviewed in Pawson 1995). In the case of the platelet derived growth factor receptor for example, autophosphorylation promotes the binding of other signalling molecules to activated SH2-sites in the intracellular domain of the receptor. Interaction with these molecules plays an important role in transmitting the receptor generated signal into the cytoplasm (Kazlauskas and Cooper 1989; Schlessinger and Ullrich 1992). How SH2 domain binding can be utilised to directly alter gene expression is best seen in the family of STAT (signal transducer and activator of transcription) proteins. Each of the STAT proteins contains both an SH2 domain and an SH2 binding site which is phosphorylated following activation of the appropriate cytokine or growth factor receptor. Reciprocal SH2 domain binding leads to the formation of STAT homo- and heterodimers. These dimers then translocate to the nucleus, where they bind to and regulate specific promoters (reviewed in Ihle and Kerr 1995).

1.2.3 Protein kinases contain a catalytic domain with conserved features

The superfamily of protein kinases is enormous, with an estimated 2000 protein kinases present in the vertebrate genome (Hunter 1994). Common to all protein kinases is the catalytic kinase domain of approximately 270 amino acids, which has been subdivided on the basis of sequence comparisons into subdomains I-XI. Dispersed among these subdomains lie at least twelve highly conserved amino acid residues required for kinase activity (Figure 1).

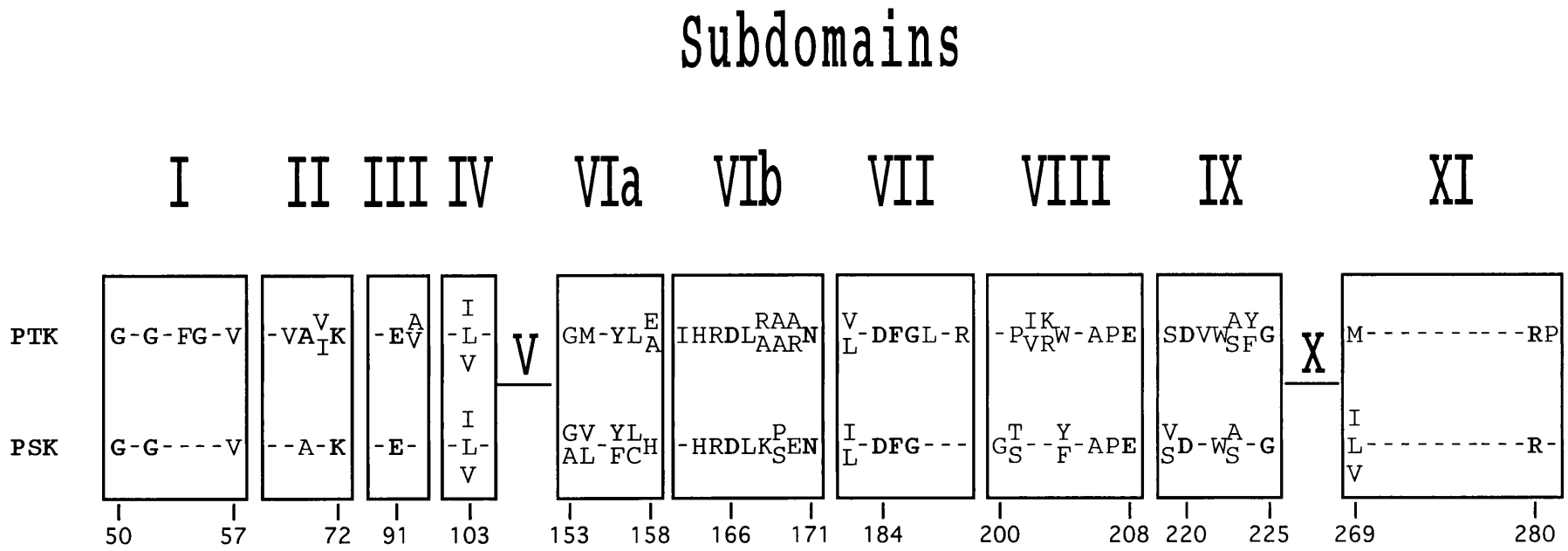


Figure 1: All protein kinases contain a catalytic kinase domain. Subdomains I-XI of the catalytic core are depicted according to Hanks *et al.*, 1988. Amino acid residues are shown in single letter code. Dash represents variabl amino acids. Certain highly conserved amino acid residues are required for complexing MgATP and mediating the phosphotransfer reaction (bold). Other amino acids are conserved in either tyrosine protein kinases (PTK) or serine/threonine protein kinases (PSK) and may be involved in determining hydroxy- amino acid specificity (dots). The enumeration of amino acids follows the amino acid sequence of cAMP-dependent kinase α.

Crystal structure analysis of the cAMP-dependent protein kinase catalytic subunit (cAPK) revealed that many of the highly conserved residues are located in loops which come together to form the active site, while variable regions located between the conserved domains lie on the outside of the globular core structure (Knighton *et al.* 1991; Bossemeyer *et al.* 1993). The globular core is subdivided into a smaller amino-terminal lobe involved in ATP-binding and a larger carboxy-terminal lobe providing catalytic centre and substrate binding sites. The glycine-rich loop of subdomain I, the conserved lysine in subdomain II and a conserved glutamine residue in subdomain III are part of the nucleotide binding lobe and serve to anchor the ATP nucleotide via its phosphate moeties. In the catalytic lobe the invariant aspartate residue of subdomain VIb is likely to act as the catalytic base, although the lysine residue of subdomain II may also be directly involved in the phosphotransfer reaction (Kamps and Sefton 1986). Other amino acid residues near the active site contributing to Mg^{2+} ATP binding and catalysis are the asparagine residue of subdomain VIb and the Asp-Phe-Gly motif of subdomain VII. Substrate recognition is likely to involve numerous residues which are not conserved among different protein kinases. Experiments using peptide analogs and chemical and mutational analysis suggest that many of the contacts are likely to be found in the carboxy-terminal regions of the catalyic domain (Gibbs and Zoller 1991).

Other regions of the kinase domain, in particular subdomains VI and VIII, contain certain amino acids which may only be conserved between subgroups of this very large family of proteins (see Figure 1). The consensus sequence IHRDL$^{R}/_{A}{}^{A}/_{R}{}^{A}/_{R}$N in subdomain VI and P$^{I}/_{V}{}^{K}/_{R}$WxAPE in subdomain VIII are predominantly found in protein tyrosine kinases (PTKs). Although the protein serine/threonine kinase (PSK) family is more diverse than the protein tyrosine kinases, the sequence motifs HRDLK$^{P}/_{S}$EN and G$^{T}/_{S}$xx$^{Y}/_{F}$xAPE are frequently found in subdomains VI and VIII of

serine/threonine kinases. These short sequence motifs have been used to predict the hydroxy-amino acid specificity of protein tyrosine (PTK) and serine/threonine (PSK) kinases, on the merit of amino acid sequence alone (Hanks *et al.* 1988).

However, a number of protein kinases have been reported that are not closely related to the sequence of any other protein kinase superfamily members. Some of these can phosphorylate both the aliphatic hydroxyl group in serine/threonine residues and the phenolic hydroxyl group in tyrosine residues. Such kinases have been labelled "dual-specificity" kinases (Lindberg *et al.* 1992). The catalytic domains of these kinases show no significant homology beyond the highly conserved residues described above. The best known members of this third "subfamily" of protein kinases are the mitogen-activated protein kinase kinases MAPKK1 and 2, also known as MEK1 and 2 (Ashworth *et al.* 1992; Crews *et al.* 1992; Kosako *et al.* 1992). As the name suggests, MAPKK is part of a protein kinase cascade, the Ras/MAP kinase signalling pathway. Upon phosphorylation by $p74^{raf}$, MAPKK becomes activated and goes on to phosphorylate MAP kinase (MAPK) on both threonine and tyrosine residues (reviewed in Marshall 1994). Interestingly, MAP kinases have also been previously suggested to be dual-specificity kinases on the basis of their ability to autophosphorylate on both tyrosine and threonine *in vitro* (Seger *et al.* 1991). However, it was not demonstrated in these studies that both activities are intrinsic to the MAP kinases by expressing a mutant kinase-negative form. In addition, all known substrates of MAPK are phosphorylated only on serine and threonine, suggesting the *in vitro* phosphorylation of tyrosine by MAPK showed by Seger *et al.* may have no *in vivo* relevance. Finally, many dual specificity kinases have a large bias for either serine/threonine or tyrosine phosphorylation of substrates. This demonstrates the importance of

determining the hydroxy-amino acid specificity of a protein kinase by studying its *in vivo* substrates (Lindberg *et al.* 1992).

The classification of protein kinase subfamilies relies only in part on the hydroxy-amino acid specificity. Other criteria include the regulators of kinase activity, such as the cAMP-dependent kinases (PKA), or the growth factor receptor kinases. These classifications are often supported by additional sequence homologies within and outside the kinase domain (Hanks *et al.* 1988; Hunter 1991). In addition, related members of a subfamily may also share other properties such as subcellular localisation and conserved functional domains. Numerous examples of such relationships are provided by the subfamily of receptor tyrosine kinases (RTK). These are distinguished from other protein tyrosine kinases by the presence of a transmembrane domain, that subdivides the molecule into an extra- and intra-cellular domain. The intracellular domain may vary in length, but always contains the catalytic kinase domain. The extracellular domain, however, exhibits great structural variability and includes diverse protein modules. This diversity may be associated with the very different ligands that activate RTK's, or may also point to other functions of RTKs, such as interactions with extracellular matrix or other moeties on the surface of adjacent cells (van der Geer *et al.* 1994). According to these different extracellular domains, the family of RTKs can be divided into several distinct classes in which each member of a particular class will share certain structural features. These structural domains are discernable in the primary amino acid sequence of the RTK's. Therefor, the characterisation of such features can be used to help predict properties of novel family members from the polypeptide sequence (Hanks *et al.* 1988)

In light of the tremendous importance of protein kinases as key regulatory molecules, the highly conserved amino acid residues of the catalytic domain have also been exploited to isolate numerous new members of this important protein super-family. This has lead to an exponential

increase in the number of known protein kinases over the past fifteen years (Hunter 1991). A frequently employed strategy involves the use of degenerate oligonucleotide primers, directed against the conserved regions of the kinase domain, in a polymerase chain-reaction (PCR) based protocol to screen for novel protein kinases (examples and variations of this approach have been reported by Wilks *et al.* 1989; Reith *et al.* 1990; Schultz and Nigg 1993).

1.3 The LIM motif defines a new regulatory domain

1.3.1 An introduction to LIM domains

Another, newly emerging family of regulatory proteins are the LIM-domain proteins (reviewed in Sanchez-Garcia and Rabbitts 1994). These proteins carry a structural motif of approximately fifty amino acids first described in three transcription factors: *lin*-11 (Freyd *et al.* 1990), *isl*1 (Karlsson *et al.* 1990) and *mec*-3 (Way and Chalfie 1988), from which the acronym LIM was derived. The consensus sequence for the LIM-domain exhibits conservatively spaced cysteine and histidine residues, arranged in a manner reminiscent of metal-binding domains found in zinc-finger proteins (Figure 2).

LIM 1	Spec	1	63
apterous	d.m.	CSGCGRQIQD . RFYLS . . . . AV . EKRWHAS CLQCYACRQP LERESSC . Y . . SRDGNIYCK ND .
CRIP	r.n.	CPKCDKEVYF AERV . . . . . TSLGKDWHRP CLKCEKCGKT LTSGG . . . HA . EHEGKPYCN HPC
cjCRP	c.j.	CGVCQKAVYF AEEV . . . . . QCEGSSFHKS CFLCMVCKKN LDSTT . . . VA VH . GDEIYCK SC .
cCRP	g.g.	CGVCQKAVYF AEEV . . . . . QCEGSSFHKS CFLCMVCKKN LDSTT . . . VA VH . GDEIYCK SC .
hCRP	h.s.	CGVCQKTVYF AEEV . . . . . QCEGNSFHKS CFLCMVCKKN LDSTT . . . VA VH . GEEIYCK SC .
enigma	h.s.	CAKCKKKI . . TGEIM . . . . . HALKMTWHVH CFTCAACKTP IRNRA . . . FY MEE . GVPYCE RD .
isl1	r.n.	CVGCGNQIHD . QYILR . . . . VSPDLEWHAA CLKCAECNQY LDESCTC . F . . VRDGKTYCK RD .
isl2	r.n.	CVGCGSQIHD . QYILR . . . . VAP . LEWHAA CLKCSECSQY LDETCTC . F . . VRDGKTYCK RD .
LH2	r.n.	CAGCGGKISD . RYYLL . . . . AV . DKQWHMR CLKCCECKLN LESELTC . F . . SKDGSIYCK ED .
Lim1	m.m.	CAGCKRPILD . RFLLN . . . . VL . DRAWHVK CVQCCECKCN LTE . . KC . F . . SREGKLYCK ND .
LIMK	h.s.	CASCGQRIYD . GQYLQ . . . . AL . NADWHAD CFRCCDCSAS LSHQ . . Y . Y . . EKDGQLFCK KD .
lin11	c.e.	CAACAQPILD . RYVFT . . . . VL . GKCWHQS CLRCCDCRAP MSM . . TC . F . . SRDGLILCK TD .
lmx1	s.h.	CEGCQRVISD . RFLLR . . . . . LNDSFWHEQ CVQCASCKEP LET . . TC . F . . YRDKKLYCK YH .
mec-3	c.v.	CNCCNEQIYD . RFIYR . . . . . MDNHSYHEN CVKCTICESP LAE . . KC . F . . WKNGRIYCS QH .
Paxillin	g.g.	CGACKKPI . . AGQVV . . . . . TAMGKTWHPE HFVCTHCGEE IGSRN . . . FF . ERDGQPYCE KD .
Pinch	h.s.	CERCKGGFAP AEHIV . . . . . QSQGELYHEQ CFVCAECFEE FPQGL . . . FY . EFEGRKYCE HD .
P-lim	m.m.	CAGCDQHILD . RFILK . . . . AL . DRHWHSK CLKCSDCHVP LAE . . RC . F . . SRRESVYCK DD .
RBTN1	h.s.	CAGCNRKIKD . RYLLK . . . . AL . DKYWHED CLKCACCDCR LGEVGSTLY . . TKANLILCR RD .
Rbtn1	m.m.	CAGCNRKIKD . RYLLK . . . . AL . DKYWHED CLKCACCDCR LGEVGSTLY . . TKANLILCR RD .
Rbtn2	m.m.	CGGCQQNIGD . RYFLK . . . . AI . DQYWHED CLSCDLCGCR LGEVGRRLY . . YKLGRKLCR RD .
RBTN3	h.s.	CAGCNRKIKD . RYLLK . . . . AL . DKYWHED CLKCACCDCR LGEVGSTLY . . TKANLILCR RD .
rCRP2	r.n.	CPKCDKTVYF AEKV . . . . . SSLGKDWHKF CLKCERCNKT LTPGG . . . HA EHDGKPFCHK PC
Xlim1	x.l.	CAGCERPILD . RFLLN . . . . VL . DRAWHVK CVQCCECKCN LTE . . KC . F . . SREGKLYCK ND .
Xlim3	x.l.	CAGCNQHIVD . RFILK . . . . VL . DRHWHSK CLKCNDCQIQ LAE . . KC . F . . SRGDSVYCK DD .
Zyxin	g.g.	CGFCRKPLSR TQPAV . . . . . RALDCLFHVE CFTCFKCEKQ LQGQQ . . . FY NVD . EKPFCE DC .
Consensus		C C * 17±1 * * Y WH F C C C * 18±1 Y FC L D C H

LIM 2		
apterous	d.m.	CSRCLASISS NELVMR . . . . AR . NLVFHVN CFCCTVCHTP LTKGDQYGI . . ID . ALIYCR TH .
cjCRP	c.j.	CPRCGQAVYA AEKV . . . . . IGAGKSWHKS CFRCAKCGKS LESTT . . . LA DKDG . EIYCK GC .
cCRP	g.g.	CPRCGQAVYA AEKV . . . . . IGAGKSWHKS CFRCAKCGKS LESTT . . . LA DKDG . EIYCK GC .
hCRP	h.s.	CPRCSQAVYA AEKV . . . . . IGAGKSWHKA CFRCAKCGKG LESTT . . . LA DKDG . EIYCK GC .
enigma	h.s.	CHGCDFKIDA GDRFL . . . . . EALGFSWHDT CFVCAICQIN LEGKT . . . FY SKK . DRPLCK SH .
isl1	r.n.	CAKCSIGFSK NDFVMR . . . . AR . SKVYHIE CFRCVACSRQ LIPGDEFAL . . REDG . LFCR AD .
isl2	r.n.	CAKCNIGFCS SDLVMR . . . . AR . DNVYHME CFRCSVCSRQ LVPGDEFSL . . RDEE . LLCR AD .
LH2	r.n.	CARCHLGISE SEMVMR . . . . AR . DLVYHLN CFTCTTCNKM LTTGDHFGM . . KD . SLVYCR LH .
lim1	m.m.	CAGCAQGISP SDLVRR . . . . AR . SKVFHLN CFTCMMCNKQ LSTGEELYI . . IDENKFVCK ED .
LIMK	h.s.	CHGCSEQITK . GLVMV . . . . A . GELKYHPE CFICLTCGTF IGDGDTYTL . . VEHSKLYCG HC .
lin11	c.e.	CAGCDGKLEK EDLVRR . . . . AR . DKVFHIR CFQCSVCQRL LDTGDQLYI . . MEGNRFVCQ SD .
lmx1	s.h.	CGGCFEAIAP NEFVMR . . . . AQ . KSVYHLS CFCCCVCERQ LQKGDEFVL . . KEGQ . LLCK GD .
mec-3	c.v.	CAGCKKGVSP TDMVYK . . . . LKAGLVFHVE CHCCSLCGRH LSPGEQILV . DDTMMTVSCM TH
Paxillin	g.g.	CYYCNGPI . . LDKV . . . . . V TALDRTWHPE HFFCAQCGVF FGPEG . . . FH EKD . GKAYCR KD .
Pinch	h.s.	CHQCGEFI . . IGRV . . . . . I KAMQQSWHPQ CFRCDLCQEV LADIG . . . F . VKQAGRHLCR PC .
P-lim	m.m.	CAACQLGIPP TQVVRR . . . . AQ . DFVYHLH CFACVVCKRQ LATGDEFYL . . MEDSRLVCK AD .
RBTN1	h.s.	CAACSKLIPA FEMVMR . . . . AR . DNVYHLD CFACQLCNQR FCVGDKFFL . . KN . NMILCQ MD .
Rbtn1	m.m.	CAACSKLIPA FEMVMR . . . . AR . DNVYHLD CFACQLCNQR FCVGDKFFL . . KN . NMILCQ VD .
Rbtn2	m.m.	CASCDKRIRA YEMTMR . . . . VK . DKVYHLE CFKCAACQKH FCVGDRYLL . . IN . SDIVCE QD .
RBTN3	h.s.	CAACSKLIPA FEMVMR . . . . AK . DNVYHLD CFACQLCNQR FCVGDKFFL . . KN . NMILCQ TD .
rCRP2	r.n.	CPRCNKRVYF AEK . . . . . V TSLGKDWHRP CLRCERCSKT LTPGG . . . HA EHDGQPYCHK PC
Xlim1	x.l.	CAGCAQGISP SDLVRR . . . . AR . SKVFHLN CFTCMMCNKQ LSTGEELYI . . IDENKFVCK ED .
Xlim3	x.l.	CAACQQGIPP TQVVRR . . . . AQ . EFVYHLH CFACIVCKRQ LATGDEFYL . . MEDSRLVCK AD .
Zyxin	g.g.	CSVCKQTI . . TDRM . . . . . L KATGNSYHPQ CFTCVMCHTP LEGAS . . . FI VDQANQPHCV DD .
Consensus		C C * 17±1 * * Y WH F C C C * 18±1 Y VC L D C H

LIM 3

Paxillin	g.g.	CGGCARAI . . LENY . . . . . I SALNTLWHPE CFVCRECFTP FINGS . . . FF EHD . GQPYCE VH .
Pinch	h.s.	CQKCHAI IDE QP . . . . . L IFKNDPYHPD HFNCANCGKE LTADARE . . . . . LKGELYCL PC .
Zyxin	g.g.	CSVCSEPIMP EPGKDETVRV VALEKNFHMK CYKCEDCGRP LSIEADENGC FPLDGHVLCM KC .
		* * * * * D
Consensus		C C **H C C C** C C H

LIM 4

Paxillin	g.g.	CSGCQKPI . . TGRC . . . . . I TAMGKKFHPE HFVCAFCLKQ LNKPT . . . FK IQN . DKPYCQ NC .
Pinch	h.s.	CGACRRPI . . EGRV . . . . . V NAMGKQWHVE HFVCAKCEKP FLGHR . . . HY ERK . GLAYCE TH .
Consensus		C C * * **WH C C C** *C C H
		F

LIM 5

Pinch	h.s.	CFHCNRVI . . EGDV . . . . . V SALNKAWCVN CFACSTCNTK LTLKN . . . KF VEFDPKPVCK KC .

Figure 2: The LIM domain consensus sequence reveals a conserved array of cysteine and histidine residues. The consensus motif was derived from twenty-four different LIM-proteins containing between one and five separate LIM domains. For some genes, known homologs from different species have been included. Multiple LIM domains within a given LIM protein are enumerated with respect to their position, beginning with the LIM domain closest to the amino-terminal. Residues involved in metal binding are highlighted in bold. Asterisks indicate the position of conserved hydrophobic residues. The single letter amino acid code has been used. Abreviations used: c.e. *Caenorhabditis elegans*, c.j. *Coturnix japonica*, c.v. *Caenorhabditis vulgaris*, d.m. *Drosophila melanogaster*, g.g. *Gajus gajus*, h.s. *Homo sapiens*, m.m. *Mus musculus*, r.n. *Rattus norvegicus*, x.l. *Xenopus laevis*. The alignment was generated using the of alignment process of Needleman and Wunsch (1970), GCG program package, University of Wisconsin/USA; gaps have been introduced to optimise the aligment.

Indeed, atomic absorption and NMR spectroscopy of various LIM-domain proteins revealed that the cysteine, histidine (and occasional aspartate residue present in the most carboxy-terminal, conserved position) can bind a total of two metal ions per LIM domain (Li *et al.* 1991; Sadler *et al.* 1992; Michelsen *et al.* 1993; Archer *et al.* 1994; Kosa *et al.* 1994). Upon tetrahedrally complexing two zinc ions, a single LIM domain can form a double-zinc finger-like structure (Michelsen *et al.* 1994), which will be discussed in more detail below.

1.3.2 LIM proteins can be divided into four different structural classes

To date, the cDNA sequences of more than twenty different LIM-domain proteins have been published, and the number is growing rapidly. Rather than relying solely on amino acid sequence homology between the LIM domains, the family of LIM-domain proteins has been subdivided into several classes based on the arrangement of LIM-domains and the presence or absence of additional protein motifs (Sanchez-Garcia and Rabbitts 1994). A schematic representation of this classification is illustrated in Figure 3.

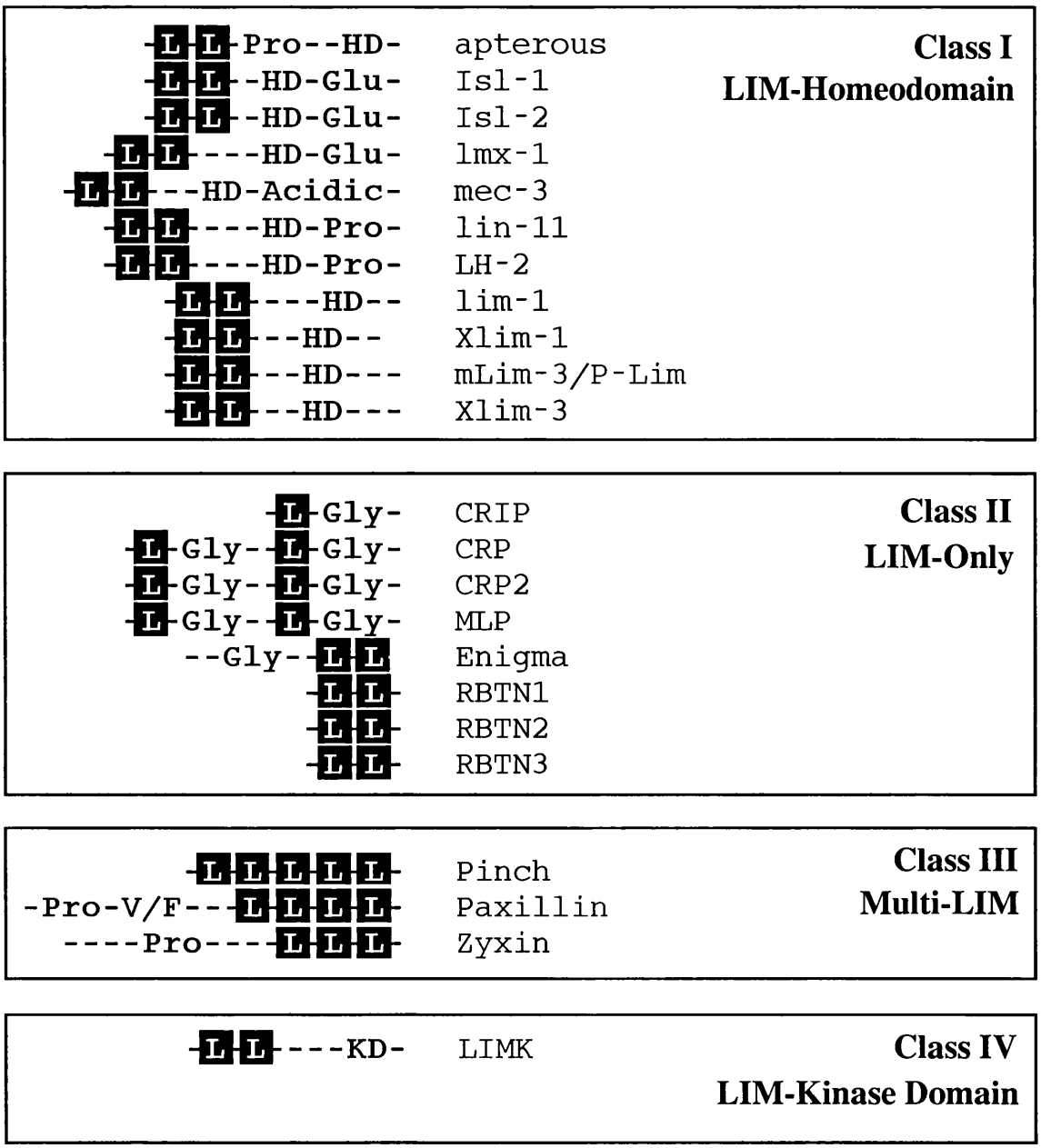


Figure 3: Classification of LIM proteins based on the number of LIM domains and additional protein domains present. At present, four classes of LIM proteins can be distinguished. The arrangement of protein domains is only schematically represented and is not drawn to scale. L, Lim domain; HD, homeodomain; KD, protein kinase domain, V/F, vinculin and pp125fak binding domain; Gly, glycine-rich sequence; Pro, proline-rich sequence; Glu, glutamine rich sequence.

1.3.3 The class of DNA binding LIM-homeodomain proteins

The first class of LIM-proteins to be identified, represents the LIM-homeodomain proteins (LIM-HD). All members of this class contain two LIM-domains arranged in tandem, and a carboxy-terminal homeodomain. The homeodomains of Isl-1 (Karlsson *et al.* 1990; Leonard *et al.* 1992), Isl-2 (Gong and Hew 1994), LH-2 (Roberson *et al.* 1994), Lmx-1 (German *et al.* 1992), mLim3/P-Lim (Seidah *et al.* 1994; Bach *et al.* 1995) and mec-3 (Xue *et al.* 1993) have been shown to bind DNA in a sequence specific manner. Some LIM-HD proteins also possess regions previously identified in the transactivating domains of transcription factors. These include acidic domains found in mec-3 (Way and Chalfie 1988), the proline rich region in lin-11 (Freyd *et al.* 1990), and the glutamine rich sequences in Isl-1, Isl-2 and Lmx-1 (Karlsson *et al.* 1990; German *et al.* 1992; Gong and Hew 1994). The presence of homeodomains suggests that members of this class of LIM proteins may act as transcriptional regulators. Indeed, a number of LIM-HD proteins have been shown to activate transcription from cell-type specific promoters: Isl-1 activates the rat insulin enhancer core (Karlsson *et al.* 1990) as well as the somatostatin promoter, in which binding of both Isl-1 and CREB are required for cell specific transcription (Leonard *et al.* 1992). Another LIM-HD protein which interacts with the insulin-1 enhancer is Lmx-1. Together with the helix-loop-helix protein Pan-1, Lmx-1 is required for the synergistic activation of insulin-1 gene transcription (German *et al.* 1992). The α-glycoprotein subunit promoter can be activated by mLim-3/P-Lim protein alone, while the prolactin and thyroid stimulating hormone β (TSHβ) promoters are synergistically activated by the binding of both mLim-3/P-Lim and the POU-domain protein Pit-1 (Bach *et al.* 1995). In *C.elegans*, mec-3 and the POU-domain protein UNC-86 are required for activation of the mec-3 promoter itself (Xue *et al.* 1993).

The involvement of LIM-homeodomain proteins in the regulation of cell-type specific genes suggests a role for this class of LIM proteins in the control of cell differentiation. This suggestion is supported by the expression pattern of numerous LIM-homeodomain proteins. One example is the recently cloned *mLim-3/P-Lim* gene (Seidah *et al.* 1994; Bach *et al.* 1995). Not only is mLim-3/P-Lim capable of activating pituitary specific gene expression (Bach *et al.* 1995), but the expression pattern of *mLim-3/P-Lim* in the developing pituitary of the mouse is also consistent with a role for mLim-3/P-Lim in the differentiation of the pituitary. In Rathke's pouch, the site at which the pituitary develops, *mLim-3/P-Lim* expression was first detected at embryonic day 9, prior to the expression of α-glycoprotein subunit, to date the earliest known marker of pituitary organ phenotype (Bach *et al.* 1995). Expression of *mLim-3/P-Lim* in the pituitary continues throughout ontogeny and suggests a role for mLim-3/P-Lim in establishing a differentiated phenotype in the pituitary cell lineage (Seidah *et al.* 1994; Bach *et al.* 1995).

An example of how the transient expression of more than one LIM homeodomain gene like *mLim*-3 may determine cell fate comes from a study on LIM homeodomain protein expression during motor neuron development in the chick spinal cord (Tsuchida *et al.* 1994). Here the combinatorial expression of *islet-1*, *islet-2*, *Lim-1* and *Lim-3* in developing motor neurons of the chick has been shown to define subclasses of motor neurons that segregate into distinct motor columns and innervate distinct targets in the periphery. This stereotyped pattern of innervation establishes a topographic neural map and is dependent on the selection of distinct axonal pathways by these subclasses of motorneurons (Tosney 1991; Eisen 1994). The fact that the expression of certain combinations of LIM-homeodomain proteins correlates with axon trajectories, suggests that this class of proteins may also be involved in determining the axonal pathways of neurons. This conclusion is supported by experiments on the axonal projections of interneurons in fruit

flies carrying a mutation of the LIM-homeodomain protein apterous (Lundgren *et al.* 1995). *Apterous* expressing interneurons are found in the ventral nerve cord of *Drosophila melanogaster* and extend axons along well defined pathways. In the absence of apterous protein, these neurons chose incorrect pathways and failed to fasciculate. Although viable, mutant flies were highly uncoordinated, which is likely to be the result of a defect in the nervous system.

A more severe phenotype has been reported for transgenic mice carrying a targeted deletion of the *Lim1* gene (Shawlot and Behringer 1995). In mouse embryos, expression of this LIM-homeodomain protein begins at embryonic day 6.5 in the prospective region of the primitive streak and is later expressed in the primitive streak and prechordal mesoderm. The majority of *Lim1*$^{-/-}$ embryos died at embryonic day 10.5. At earlier stages *Lim1*$^{-/-}$ embryos completely lacked anterior head structures while the remaining body axis had developed normally. When the formation of the node and primitive streak were analysed, it became clear that *Lim1* is essential for the formation of an early node and anterior axial mesoderm. As an important regulator of the head organiser in vertebrates, *Lim1* is required for the formation of all head structures anterior to the otic vesicle.

Direct evidence for the involvement of LIM-HD genes in differentiation and cell determination, comes from the nematode genes *lin-11* and *mec-3*. Null mutants of *lin-11* lead to the loss of assymetric cell division in the secondary vulval precursor cells, thus resulting in sterile animals (Freyd *et al.* 1990). A similar disruption of assymetric cell division occurs in the mechanosensory neuron lineage of *mec-3* mutant animals (Way and Chalfie 1988; Way *et al.* 1992). In summary, LIM-homeodomain proteins are transcription factors, that control differentiation at several different levels of development.

1.3.4 The class of LIM-domain-only proteins includes two oncogenes

The second major class of LIM-proteins contains one or two LIM-domains, but no other structural motifs with a known function. The majority of proteins in this class are associated with cytoskeletal components (Sadler *et al.* 1992; Crawford *et al.* 1994). These include the cysteine rich proteins CRP (Liebhaber *et al.* 1990; Wang *et al.* 1992; McLaughlin *et al.* 1994) and CRP2 (Okano *et al.* 1993). Interestingly, all CRP mRNAs contain a motif responsible for the rapid degradation of mRNA. This motif is frequently found in immediate early genes and like these the expression of CRP is rapidly induced by serum (Wang *et al.* 1992; McLaughlin *et al.* 1994). CRIP, a small protein with only one LIM domain, has been implicated in zinc transport in intestinal cells and is likely to be associated with the cell membrane (Birkenmeier and Gordon 1986; Hempe and Cousins 1991).

The other group of LIM-only proteins comprise the rhombotins RBTN-1/Ttg-1 (McGuire *et al.* 1989; Boehm *et al.* 1990), RBTN-2/Ttg-2 (Boehm *et al.* 1991; Royer-Pokora *et al.* 1991) and RBTN-3 (Boehm *et al.* 1991; Foroni *et al.* 1992) and the muscle LIM protein, MLP (Arber *et al.* 1994). Unlike the cysteine-rich proteins, these are located in the nucleus and have been proposed to act as transcriptional regulators (Boehm *et al.* 1990; Rabbitts and Boehm 1990; McGuire *et al.* 1991). In fact, RBTN-1 and RBTN-2 have been shown to specifically bind to TAL1, TAL2 and LYL2, three helix-loop-helix proteins implicated in T-cell acute lymphoblastic leukemia (T-ALL) (Valge-Archer *et al.* 1994; Wadman *et al.* 1994). The interaction between RBTN-1/-2 and these helix-loop-helix proteins is of special interest as both RBTN-1 and 2 were originally isolated from chromosomal breakpoints in patients with T-cell acute leukemias. The ability of RBTN-1 and 2 to induce tumours was confirmed in transgenic mice expressing either protein from a T-cell specific promoter (Fisch *et al.* 1992; Larson *et al.* 1994). Conversely, the disruption of the RBTN-2 gene in transgenic mice results in an embryonic lethal

phenotype in which the differentiation of erythroid cells is completely abolished (Warren *et al.* 1994). Therefore, while RBTN2 is essential for normal erythropoesis, it may also contribute to the formation of T-cell acute leukemia. To date, RBTN-1/Ttg-1 and RBTN-2/Ttg-2 are the only known LIM-domain containing oncogenes.

The only other LIM-only protein found in the nucleus is the muscle LIM protein, MLP. Accumulation of MLP in the nucleus is observed in C2 myoblasts which have been induced to differentiate by serum withdrawal. While overexpression of MLP does not induce muscle differentiation in non-myogenic cell types, it does potentiate differentiation of C2 myoblasts. Together with the expression of MLP in mesoderm, heart and striated muscle, the ability of MLP to promote C2 myoblast differentiation suggests a role for MLP in muscle cell differentiation (Arber *et al.* 1994).

1.3.5 The Multiple-LIM proteins represent a class of adaptor proteins

The third class of LIM-proteins comprises proteins with three or more LIM-domains. Pinch, a protein consisting almost entirely of LIM domains, was cloned on the basis of an autoepitope in the loop of the third LIM domain. Such autoepitopes are recognised by autologous immunoglobulins and have been implicated in the removal of senescent red blood cells by phagocytosis (Rearden 1994). However, it remains unclear whether this represents the physiological function of Pinch. Depending on the presence of other functional domains in Pinch, this protein may also be considered a member of the LIM-only class of LIM proteins.

The two other members of this class, zyxin and paxillin, are cytoskeletal proteins found in focal adhesion complexes. In contrast to the LIM-HD proteins, the LIM domains of both zyxin and paxillin are located at the carboxy-terminal end of the molecule. The amino-terminal half of zyxin consists of a very proline-rich region with stretches of poly-prolines

reminiscent of SH3 domain binding sites. However, this region has been shown to bind α-actinin which in turn binds actin, vinculin and β integrins, all components associated with the cytoskeleton. The C-terminal half of zyxin has been shown to bind cCRP *in vitro* (see section 1.3.8, Schmeichel and Beckerle 1994) and zyxin and cCRP are found to be colocalised in chicken embryo fibrolasts (Sadler *et al.* 1992). Paxillin also interacts with a variety of different proteins, including vinculin, talin and a number of protein kinases (Turner and Miller 1994). None of these interactions have been mapped to the four LIM domains. Instead, an amino-terminal region mediates binding of both vinculin and pp125^{fak}, the focal adhesion kinase (Turner and Miller 1994). This interaction is distinct from the binding of pp60^{src} to the SH2 and SH3 binding sites of paxillin (Weng *et al.* 1993). The activated SH2 binding site is also a target for Crk, an SH2-, SH3-domain containing adaptor molecule capable of transforming chicken embryo fibroblasts (Birge *et al.* 1993). Disruption of the SH2 domain in Crk abolishes the transforming capacity of *v-crk* (Mayer and Hanafusa 1990). In myeloid cells, human paxillin appears to be associated with the p210$^{BCR/ABL}$ oncogene, although the binding site has not been mapped (Salgia *et al.* 1995). Therefore, paxillin may serve as an adaptor protein, capable of bringing together other proteins in a multimeric complex. As some of these components are part of the signal transduction machinery (pp60src, pp125fak, c-crk) paxillin may help to mediate integrin-triggered signal transduction, leading to changes in the structure of the cytoskeleton (Schaller and Parsons 1995)

1.3.6 The latest in LIM proteins: a LIM-kinase domain protein

The amino acid sequence of a recently cloned human gene, the putative LIM motif-containing protein kinase LIMK (Mizuno *et al.* 1994), predicts the presence of both LIM- and kinase domains within the same molecule. Similar to the LIM-HD proteins, the two LIM domains are arranged in tandem at the

amino-terminal end, while the presence of a short basic motif suggests that LIMK protein may be targeted to the nucleus. Although kinase activity has not yet been demonstrated for LIMK, the discovery that LIM proteins may also contain a key element of the signal transduction machinery not only defines a new class of LIM proteins, but also opens up new possibilities for the regulatory potential of LIM domain proteins. The question regarding the function of the LIM domain itself will be addressed in the next two sections.

1.3.7 LIM domains represent a new class of zinc-fingers

The conserved spacing of cysteine and histidine residues found in LIM-proteins is reminiscent of cysteine/histidine motifs in zinc-finger proteins (McGuire *et al.* 1989; Freyd *et al.* 1990; Li *et al.* 1991). The zinc-finger motif was first identified in the transcription factor IIIA of *Xenopus* (Ginsberg *et al.* 1984). This DNA and RNA binding protein contains nine tandem repeats of a thirty amino acid sequence unit, each of which contains a sequence motif of the form: Cys-x_{2or4}-Cys-x_{12}-His-x_4-His, where x denotes any amino acid residue (Brown *et al.* 1985; Miller *et al.* 1985). Structure analysis of a TFIIIA-type zinc finger unit reveals the formation of an antiparallel ribbon and an α helix, while the conserved cysteine and histidine residues bind in a tetrahedral arrangement to a single zinc atom (Berg 1988). This brings together the two ends of the protein domain to form a compact domain with a hydrophobic inner core, while amino acids from the amino-terminal part of the α-helix make contact with the major groove of the DNA double helix (Pavletich and Pabo 1991). Therefore, the conserved cysteine and histidine residues provide the framework of tertiary folding, while variable residues are likely to mediate specific interactions.

It is interesting to note that the intron-exon boundaries in the TFIIIA gene mark most of the finger domain boundaries (Miller *et al.* 1985; Tso *et al.* 1986), an observation which supports the model that zinc-fingers are

independently folded protein domains. Amongst the LIM-proteins for which the genomic structure has been studied, this also is the case for both LIM domains in RBTN2, but only for LIM domain 1 in RBTN1 and 3 (Foroni *et al.* 1992), while hCRP shows no alignment of exon and LIM domain boundaries (Wang *et al.* 1992).

Similar zinc-finger motifs have since been identified in a large number of DNA binding proteins. Depending on the spacing and the usage of either cysteine or histidine residues, these can be grouped into five major classes: the TFIIIA class, the GAL4 class, the steroid receptor class, the RING-finger class and the GATA-1 zinc finger class (Figure 4).

Zinc-Finger Prototype	Arrangement of metal-binding amino acid residues
Transcrition Factor IIIA	C x_2 C -x_{12}- **H** x_3 **H** or C x_4 C -x_{12}- **H** x_3 **H**
Glucocorticoid Receptor	C x_2 C -x_{13}- C x_2 C-x_{15}- C x_5 C -x_9- C x_2 C x_4 C
GAL4	C x_2 C -x_6- C -x_6- C x_2 C -x_6- C
GATA-1	C x_2 C -x_{17}- C x_2 C-x_{30}- C x_2 C -x_{17}- C x_2 C
RING 1	C x_2 C -x_{12}- C x_2 **H**-x_2- C x_2 C -x_{11}- C x_2 C
LIM	Ⓒ x_2Ⓒ -$x_{17\pm1}$- **H** x_2 C-x_2- Ⓒx_2Ⓒ -$x_{18\pm1}$- C x_2 C

Figure 4: Conserved metal-binding residues define the zinc-finger domain. The conserved motifs and number of zinc atoms bound by each motif are presented for different classes of zinc-finger type proteins. Residues which are conserved in all LIM proteins are circled. C, cysteine; H, histidine; D, aspartate; x, any amino acid residue. (with modifications from Sanchez-Garcia and Rabbitts 1994) .

Each of the cysteine-rich motifs shown in Figure 4 is capable of complexing zinc. TFIIIA (Frankel *et al.* 1987; Berg 1988), steroid receptors

(reviewed in Schwabe and Rhodes 1991), GAL4 (Marmorstein *et al.* 1992) and GATA-1 (Omichinski *et al.* 1993) are known to contain α-helices which participate in sequence specific DNA binding. Although the structure of the RING1 domain has not yet been resolved, the fact that this motif has been detected in a large number of DNA binding proteins indicates that the RING1 zinc finger may also be involved in DNA binding (Lovering *et al.* 1993).

When compared to known zinc-finger consensus motifs, the LIM-domain consensus sequence is most similar to the RING1 and GATA-1 type zinc-fingers (Sanchez-Garcia and Rabbitts 1994). As with GATA1 and RING1 motifs, two zinc atoms are complexed per LIM domain. While the short, two amino acid linker between the zinc coordination sites of LIM domains is also present in RING1 domains, the length of zinc-finger loops resembles more closely that of the GATA1 domain. Unlike GATA1, both LIM domains (CCHC) and RING1 zinc-fingers (CCCH) utilise histidines to bind metal ions. The recently published LIM-protein Pinch also contains a CCHH-type zinc-finger (Rearden 1994). Recently, the structure of the carboxy-terminal LIM domain from hCRP has been analysed by nuclear magnetic resonance spectroscopy (Perez-Alvarado *et al.* 1994). Like the GATA1 zinc finger, the hCRP LIM domain complexes two zinc atoms and forms two antiparallel β-sheets and an α-helix. While the overall structure of the LIM zinc-finger is reported to be unique, the carboxy-terminal CC-CC module, which contains the α-helix has a striking structural similarity to equivalent regions of the GATA1 and steroid receptor, which mediate DNA binding. This has led to the suggestion that the carboxy-terminal zinc-finger may be capable of binding DNA, while the amino-terminal finger may be involved in protein-protein interactions (Perez-Alvarado *et al.* 1994).

1.3.8 Are LIM domains multifunctional binding interfaces?

Because of the similarities between zinc-fingers that mediate DNA binding in nuclear transcription factors, and the double zinc-finger/LIM motif, it has been suggested early on that LIM domains may also be involved in DNA binding (Freyd *et al.* 1990; Karlsson *et al.* 1990). However, to date no evidence has been published which would support such a function for LIM-domains. Instead, several lines of research have pointed towards a possible role for LIM domains as protein binding modules (Rabbitts and Boehm 1990; Sadler *et al.* 1992). The first experimental evidence of LIM domains involved in protein-protein binding came with the isolation of a zyxin binding protein, cCRP. Like zyxin, cCRP is also a LIM-protein. Both zyxin and cCRP co-localised to stress fibers and adhesion plaques in chicken fibroblasts, while recombinant hCRP bound to zyxin *in vitro* (Sadler *et al.* 1992). This suggested a possible role for LIM domains in protein binding, which in this case might serve to localise these proteins to the cytoskeletal compartment. Further evidence for a role of LIM-domains in protein-protein interactions comes from studies on the activity of LIM-homeodomain transcription factors. One such factor, lmx-1 binds to the FLAT element of the insulin 1 enhancer and is capable of synergising with the helix-loop-helix protein Pan-1 to activate insulin 1 transcription in BHK cells (German *et al.* 1992). This synergistic activation did not involve changes in DNA binding activity. While the LIM-domains where required for the synergistic activation, a direct interaction between Lmx-1 and Pan-1 was not shown. Nor did a truncated form of Lmx-1 containing only the LIM domains (without the attached homeodomain) interfere with the synergistic activation (German *et al.* 1992).

Evidence for a direct interaction between LIM domains and other protein domains has recently been reported from a number of laboratories. Prime candidates as mediators of protein-protein interactions are members of

the LIM-only class of proteins, in particular the nuclear proteins RBTN-1/Ttg-1 (McGuire *et al.* 1989; Boehm *et al.* 1990) and RBTN-2/Ttg-2 (Boehm *et al.* 1991; Royer-Pokora *et al.* 1991). On the basis of their nuclear localisation (McGuire *et al.* 1991), the absolute requirement for RBTN-2 in erythroid development (Warren *et al.* 1994), the highly regulated expression pattern of RBTN-1 (Greenberg *et al.* 1990) and the ability of both RBTN-1 and 2 to induce T-cell acute lymphoblastic lymphoma in transgenic mice (Fisch *et al.* 1992), RBTN1 and 2 were postulated to be transcriptional activators. Indeed, a direct interaction between RBTN-2 and the helix-loop-helix transcription factor TAL-1 was demonstrated in erythroid cells by immunoprecipitation (Valge-Archer *et al.* 1994). This was confirmed by using the two-hybrid system (Fields and Song 1989) to demonstrate a highly specific interaction between RBTN-1 and 2 with the helix-loop-helix domains of TAL1, TAL2 and LYL1 in Jurkat cells (Wadman *et al.* 1994). Since the RBTN proteins are mostly comprised of LIM domain sequences, it is likely that one of the two double zinc-finger domains mediates this protein-protein interaction. This was taken one step further by demonstrating the ability of RBTN1 or 2, TAL1 and another helix-loop-helix factor, E47 to form a heterotrimeric complex *in vivo* (Wadman *et al.* 1994). These experiments confirm a role for LIM domains in mediating protein-protein binding. This also shows that RBTN-1 and 2 may act as transcriptional regulators through association with basic helix-loop-helix transcription factors.

A more detailed analysis of the protein binding requirements of LIM-domains comes from recent experiments involving the two cytoskeletal proteins hCRP and zyxin. Using a filter overlay binding assay, and various combinations of recombinant hCRP peptide fragments, Feuerstein *et al.* (1994) demonstrated the ability of a minimal LIM domain (carboxy-terminal LIM domain lacking the glycine-rich sequence) to bind to itself or full length

hCRP. The two-hybrid system was used in NIH 3T3 fibroblasts to further delineate the minimum requirements for LIM-LIM binding. By substituting the first, last or both first and last zinc-binding cysteine residues in the minimal LIM domain with serine, zinc binding to either the first, second or both zinc fingers was disrupted. Using these constructs, it was shown that the zinc-finger doublet is necessary to mediate protein-protein binding and that it is dependent on the coordinate binding of two zinc atoms (Feuerstein *et al.* 1994). Similarly subcloned regions of the multi-LIM protein zyxin were tested for the ability to bind to cCRP in a blot overlay assay *in vitro* (Schmeichel and Beckerle 1994). While the amino-terminal, proline-rich domain of zyxin bound α-actinin, another cytoskeletal component, the LIM domains were required for cCRP binding. In fact, out of the three LIM domains present in zyxin, domain 1 was sufficient and necessary to bind cCRP in a specific and saturable manner. LIM domains 2 and 3 of zyxin did not exhibit any cCRP binding activity, leaving the question with respect to a possible function for these two carboxy-terminal LIM domains unanswered. However, these experiments clearly demonstrate the ability of a LIM domain protein like zyxin to act as a potential adaptor molecule with multiple protein-binding interfaces.

Another type of protein domain with which LIM domains can interact is the POU-domain. Like helix-loop-helix domains, POU domains are primarily found in DNA-binding transcription factors and can participate in protein dimerisation (Treacy *et al.* 1991). Pit-1 is one such POU-domain protein, and is required for the activation of a number of peptide hormone promoters in the pituitary gland, including the prolactin (Prl) and thyroid-stimulating hormone β (TSHβ) promoter. mLim-3/P-Lim, a LIM-homeodomain protein, is also expressed in the pituitary gland and has recently been shown to activate the Prl and TSHβ promoters, together with Pit-1, in a synergistic manner (Bach *et al.* 1995). *In vitro* binding assays using truncated forms of

recombinant mLim-3/P-Lim and Pit-1 peptides revealed that either LIM domain of P-Lim was sufficient to mediate binding to the POU-doman of Pit-1, while both POU-specific and POU-homeodomain were needed for efficient binding to mLim-3/P-Lim (Bach *et al.* 1995).

Finally, in a screen for proteins that are capable of recognising the endocytic codes of cell surface receptors, an unusual LIM protein was isolated, aptly named Enigma (Wu and Gill 1994). A key feature of the endocytic code is the presence of a tyrosine containing tight turn. Using a yeast two hybrid system and expression contructs encoding various LIM domain deletion mutants, the ability of Enigma to specifically bind the insulin receptor endocytic sequence was mapped to the carboxy-terminal LIM domain. A synthetic peptide sequence previously shown to function as a strong endocytic signal was bound by both LIM domains independently, and also by the carboxy-terminal LIM domain of mec-3. Whether these interactions reflect a physiological role for Enigma, or mec-3, remains to be seen (Wu and Gill 1994).

In summary, the ability of LIM domains to bind to other LIM domains, helix-loop-helix domains, POU domains and even tyrosine-containing tight turns, clearly establishes this new double zinc-finger module as a protein-protein binding interface. In this respect LIM domains are similar to other protein dimerisation motifs found in transcription factors. These motifs include the leucine zipper (Landschulz *et al.* 1988; Turner and Tjian 1989), the helix-loop-helix motif (Murre *et al.* 1989; Murre *et al.* 1989; Cole 1991), and the POU-homeodomain (Stern *et al.* 1989; Ingraham *et al.* 1990). More recently, the PAS motif has been identified in the *period* gene of *D. melanogaster*. This domain mediates dimerisation with other PAS containing proteins, some of which are not transcription factors (Huang *et al.* 1993). However, unlike these binding motifs, LIM domains can interact with LIM-domains *and* other types of protein-protein interfaces. Only the Rel/dorsal

homology region of NF-kB related proteins exhibits a similar diversity. In p50 NF-kB this region is required for both DNA binding and factor dimerisation, while the p105 NF-kB precursor also contains ankyrin-like repeats capable of binding the Rel homology region (reviewed in Blank *et al.* 1992)

Although zinc finger domains are generally considered to be DNA binding domains, LIM-type zinc fingers are not the only example of zinc-finger-like structures which can mediate protein binding. While the zinc finger region in the CR1 domain of Raf may contribute to the binding of Raf to the annexin-like region of 14-3-3 proteins (Freed *et al.* 1994), the cysteine-rich C1 region of protein kinase C (PKC) appears to mediate binding to phorbol esters (Kaibuchi *et al.* 1989; Ono *et al.* 1989; Burns and Bell 1991). Whether this putative double-finger domain of PKC is also involved in the binding of PKC to intracellular receptors for activated C-kinase (RACKs) is not yet clear (Mochly-Rosen *et al.* 1991; Ron *et al.* 1994). In addition the protein interactions between E1A and the TATA binding protein (Geisberg *et al.* 1994), and between the initiator binding protein YY1 and the Sp1 transcription factor (Lee *et al.* 1993) have been attributed to the zinc finger-like domains of E1A and YY1.

It is interesting to note that the homodimeric binding of the GATA-1 transcription factor was recently shown to be mediated by a region overlapping with the carboxy-terminal α-helix of the second zinc-finger (Crossley *et al.* 1995). As this region of the GATA-1 zinc-finger has also been implicated in DNA binding, Crossley *et al.* have suggested that this region may represent a multifunctional domain. Similarly, the structural homology of this region between GATA-1 and LIM-type zinc fingers has raised the possibility that the LIM domain may be able to bind both protein and DNA molecules (Perez-Alvarado *et al.* 1994). Should this be the case, it

would add another degree of complexity to an already highly versatile protein domain.

1.4 The scope of this thesis

One central research interest in the laboratory of Professor Mark Noble has been to study the mechanisms controlling the division and differentiation of oligodendrocyte-type 2 astrocyte (O-2A) precursor cells both during development and in the adult animal (Raff *et al.* 1983; Noble and Murray 1984). The work presented here describes the cloning and preliminary characterisation of a novel murine gene, *Limk1*, which contains both LIM and protein kinase domains and which is also expressed in cells of the O-2A lineage (Pröschel *et al.* 1995).

2

Methods & Materials

2.1. Methods

2.1.1 Small scale DNA isolation using alkaline lysis

a) Bacteria Culture:
A single colony was picked from an agar plate and transferred to a replica plate. The same colony was used to inoculate 1.5 ml Super Broth Media. The culture was then incubated overnight in a shaker at 37°C.

> *Super Broth*
> 32g Bacto-Tryptone (Gibco), 20g Yeast extract (Gibco), 10g Sodium Morpholinopropansulfate (MOPS-Na, Merck) completed to 1 litre with H_2O

b) DNA Isolation:
The bacterial suspension was centrifuged at 13000 rpm for 40sec. and the supernatant was aspirated. The pellet was mixed with 100µl Solution I and incubated for 5 min. at room temperature. 200µl Solution II were added and mixed by inverting gently until the solution appeared clear. This mixture was incubated for 5 min. on ice before 150µl cold Solution III were added to the suspension. The mixture was spun at 13000 rpm for 10 min. at 4°C and the supernatant was transferred to a fresh tube.

The supernatant was mixed with 0.6 volume isopropanol and centrifuged at 8000 rpm for 10 min. at room temperature. The upper phase was transferred into a fresh microcentrifuge tube, mixed with 700µl 2M NH_4Ac and spun at 13000 rpm for 5 min. at 4°C. The supernatant was transferred to a fresh tube and extracted with 400µl isopropanol. After spinning at 13000 rpm for 5 min. at room temperature, the precipitate was washed with 75% (v/v) ethanol, precipitated again and the DNA pellet was dissolved in 100µl TE buffer.

> *Solution I:*
> 50mM Glucose (Sigma), 10mM EDTA (Sigma), 25mM Tris, HCl pH 8.0 (Merck).
> Add powdered lysozyme (Sigma)to 4mg/ml just before use.

Solution II:
0.2M NaOH (Merck); 1% (w/v) SDS (Merck)

Solution III:
3M KAcetate (Merck), 2M Glacial Acetic Acid (Merck)

TE Bufffer
10mM Tris HCl, pH7.5 (Merck), 1mM EDTA, pH8 (Sigma)

c) RNAse Treatment:
For use a template in sequencing reactions, the RNA was removed by digestion with RNase A. The DNA was resuspended in 100µl TE buffer before adding 1µl 20mg/ml RNAse A and incubating the mixture at 37°C for 30 min. The sample was then mixed with 60µl 20% (w/v) PEG_{6000}/2.5M NaCl (Merck) and incubated for 1 hr on ice. After precipitating the DNA at 13000 rpm for 15min. at 4°C the pellet was dissolved in 100µl TE buffer and twice extracted with CIA. The final precipitation was carried out in 10µl 3M NaOAc and 60µl isopropanol. After spinning for 10 min. at 13000 rpm the DNA pellet was washed in 75% (v/v) ethanol and dissolved in 100µl TE buffer.

2.1.2 Small scale DNA isolation using CTAB

a) Bacteria Culture:
A single colony was picked from an agar plate and transferred to a replica plate. The same colony was used to inoculate 1.5 ml Super Broth Media. The culture was then incubated overnight in a shaker at 37°C.

Super Broth
32g Bacto-Tryptone (Gibco), 20g Yeast extract (Gibco), 10g Sodium Morpholinopropansulfate (MOPS-Na, Merck) completed to 1 litre with H_2O

b) DNA Isolation:
The bacterial suspension was centrifuged in a 1.5ml microcentrifuge tube for 30 seconds at 13000 rpm and the pellet was incubated for 5 min. at room temperature with 200µl STET Buffer and 4µl lysozyme [50 mg/ml]. Bacterial debris was denatured by heating the suspension at 95°C for 45 seconds After spinning for 10 min. at 13000 rpm the bacterial debris, visible as a slimy pellet, was removed with a sterile yellow tip. To the remaining volume (ca. 100-150µl) 8µl CTAB [5% w/v] were added to precipitate the DNA. The DNA was pelleted at 13000 rpm for 5 min. and dissolved by gently vortexing in 300µl 1.2M NaCl. The DNA was reprecipitated with 750µl 100% ethanol and washed with 500µl 70% (v/v) ethanol. The DNA pellet was dried and

dissolved in 20µl TE buffer. This procedure gave an average yield of ca.5µg plasmid DNA (Stratagene Bluescript Vector in HB101).

STET-Buffer:
8% (w/v) Sucrose (Sigma), 0.1% (v/v) Triton X-100 (Sigma), 50mM EDTA (Sigma), 50mM Tris-HCl/pH 8.0 (Merck)

CTAB:
5% (w/v) Cetyl Trimethyl Ammonium Bromide (Sigma)

TE Bufffer
10mM Tris HCl, pH7.5 (Merck), 1mM EDTA, pH8 (Sigma)

c) Preparing DNA for Sequencing:
To denature the DNA 2µl 2M NaOH and H_2O were added to bring the reaction volume to a total of 20µl. The DNA was incubated for 20 min. at 68°C. The denatured DNA was mixed with 8µl 5M NH_4OAc/pH 5.4. The DNA was precipitated with 100µl 100% ethanol for 5 min. on dry ice and washed with 100µl 70% (v/v) ethanol. The DNA pellet was dried in a speedvac for 5 min. DNA thus prepared is suitable for dideoxynucleotide sequencing.

2.1.3 Large scale DNA isolation using CsCl-gradient centrifugation

a) Bacteria Culture
500ml of LB Medium (Gibco) containing antibiotic were inoculated with 25ml late log phase bacterial culture. The bacteria culture was amplified to an OD_{600} 0.4 (ca. 2.5 hours) and chloramphenicol was added to a final concentration of 150µg/ml. The bacteria suspension was pelleted at 6000 rpm for 10 min. at 4°C in a Sorvall GSA Rotor, the pellet was dissolved in 100ml ice cold STE buffer and again pelleted at 6000 rpm for 10min. at 4°C. The supernatant was discarded and the pellet well drained.

b) Lysis by Alkali
The bacterial pellet was resuspended in 40ml Solution I, mixed well and lysed with 5mg/ml lysozym for 10 min. at room temperature. To denature the lysate 80ml Solution II were added for 10 min. at room temperature. 40ml Solution III were mixed well into the solution and incubated for 10 min. to 1 hr on ice. The denatured bacterial lysate was pelleted at 10000 rpm for 20 min. at 4°C and the supernatant was filtered through one layer of gauze, or Whatmann No.1 filter paper into fresh GSA centrifugation cups. The DNA was mixed with 0.6 volumes isopropanol and immediately spun at 9000 rpm for 10 min. at room temperature. The pellet was rinsed carefully with 100% ethanol and resuspended in 7 ml TE-50 buffer.

STE buffer:
0.1M NaCl (Merck), 10mM Tris, HCl pH 8 (Merck), 1mM EDTA, pH 8 (Sigma)

Solution I: (cold)
50mM Glucose (Sigma), 10mM EDTA 25mM (Sigma), Tris, HCl pH 8.0 (Merck). Add powdered lysozyme to 4mg/ml just before use.

Solution II: (fresh)
0.2M NaOH (Merck), 1% (w/v) SDS (Merck)

Solution III:
3M Potassium Acetate (Merck), 2M Glacial Acetic Acid (Merck)

TE-50 buffer:
50 mM Tris-HCl pH 8 (Merck), 50mM EDTA-Na pH 8 (Sigma)

c) CsCl/EtBr Gradient Centrifugation:
In a 50ml tube, 9g CsCl (Merck) were mixed with 8.63g DNA solution (in TE-50) to obtain a refraction index of 1.39. Ethidium bromide (Sigma) was added to a final concentration of 9g/17.1g DNA solution. The solution was spun at 4000 rpm for 20 min. at room temperature and the supernatant was transferred into Ti70.1 tubes suitable for a fixed angle rotor. The tubes were topped up with a solution containing 1.58 g/ml CsCl in TE-50 and spun at 55000 rpm for 20 hours at 20°C. The fixed angle provided a shallow gradient with an upper band showing chromosomal DNA, a lower band containing plasmid DNA and a pellet representing RNA/ethidium bromide complexes. The plasmid band was removed and transferred into VTi.65 tubes suitable for a vertical rotor, filled to the top with 1.58 g/ml CsCl in TE-50 Solution and centrifuged at 65000 rpm for 4 hours at 20°C or at 30000 rpm overnight. The plasmid band was removed and the ethidium bromide was washed out by repeatedly adding 2 volumes of 1-Butanol/H_2O until the solution appeared colourless. The plasmid DNA was mixed with 3 volumes TE-10 and 0.6 volumes Isopropanol, incubated for 2hours at -20°C and precipitated at 12000 rpm for 20 min. The DNA pellet was washed with 70% (v/v) ethanol and resuspended in 200µl TE buffer. This purification procedure yielded on average 3-5 mg plasmid DNA/500 ml bacteria culture. The DNA was stored at 4°C to avoid frequent freeze/thaw cycles.

1-Butanol/ H_2O:
Add 1 volume 1-Butanol (Merck) to 1 volume H_2O, let phases separate.

TE Bufffer
10mM Tris HCl, pH7.5 (Merck), 1mM EDTA, pH8 (Sigma)

2.1.4 λ Phage DNA isolation

a) Plating:
Fresh 15cm Ø NYZM/NYZCM agarose plates were prepared and pre-warmed at 37°C. 1ml bacteria overnight culture was gently mixed with 50-200µl Phage diluent (15ml test tube) for 15 min. Top Agarose (0.7% (w/v) agarose in NYZM medium, 6.5ml/15cm Ø dish) was heated in a microwave oven until molten and allowed to cool to 42°C in a waterbath. The Top Agarose was added to the phage/bacteria mix, mixed quickly and poured onto the prewarmed plates. Plates were incubated overnight at 37°C.

NZY Medium [21g/l]
21g/l NZY Medium (Gibco), 0.2mM $MgSO_4$ (Merck), 1% (w/v) Agarose (Gel grade), mixture is autoclaved and poured onto plastic dishes.

b) Phage Elution:
To each plate 2ml TM were added and incubated for 1-2hours at 37°C or 12hours at 4°C. The TM solution was transferred to 12ml snap-cap tubes, the plates rinsed with an additional 1ml of TM/plate and combined with the first wash. The solution was spun at 4000g for 10min. at 4°C to remove bacterial debris.

TM (λ diluent):
10mM Tris HCL (pH7.2), (Merck) 10mM $MgSO_4$ (Merck)

c) DNA Preparation
The supernatant was transferred to a fresh snap-cap tube and incubated with 1µl RNase A [1mg/ml] and 1µl DNAse [1mg/ml] for 15min. at 37°C. The digested supernatant solution was vortexed with 3ml Phage Precipitation Solution and incubated for 1-2 hours on ice. After centrifugation at 10000g at 4°C (Sorvall, HB-4) the supernatant was aspirated and any remaining fluid was allowed to drain (invert tube). The pellet was dissolved in 0.5ml TE (pH8), transferred to microcentrifuge tubes and the DNA was extracted using equal volumes of PCIA and CIA . The DNA was precititated in $^1/_{10}$ volume of 3M NaOAC (pH5) and 2 volumes of 100% ethanol on dry ice for 30 min. DNA was pelleted at 15000 rpm for 15min. at 4°C and dissolved in 50µl TE buffer.

Phage Precipitation Solution
20% (w/v) PEG 8000 (Merck), 2M NaCl in TM Buffer

TE Bufffer
10mM Tris HCl, pH7.5 (Merck), 1mM EDTA, pH8 (Sigma)

2.1.5 Isolation of genomic DNA from tissue

a) DNA Isolation:
Tissue was dissected and rinsed in cold 1x PBS. The washed tissue was chopped into small pieces and snap-frozen in 12ml snap cap tubes in liquid nitrogen. Percussion mortars were cooled in liquid N_2/dry ice and the frozen tissue pieces were filled into the mortar. Tissue samples were fractured with several hammer strokes. The ground tissue was transferred into a 50ml test tube. To 100mg tissue, 1ml Lysis Buffer and $^1/_{100}$ volume of Proteinase K [20mg/ml] was added. This mixture was incubated overnight at 53°C. To digest RNA $^1/_{100}$ volume of RNase A [25mg/ml] was added and incubated for 1-2 hours at 37°C. The DNA was extracted with an equal volume of phenol (equilibrated with TE-10, pH7.5) for a few hours by gently rotating. The suspension was spun at 1000 rpm for 5 min. This phenol extraction was repeated twice followed by one extraction using an equal volume of chloroform/isoamylalkohol (96:4) . The final extract was precipitated in 4% (w/v) NaOAc and 2 volumes 100% ethanol for 20 to 60 min. on ice. The clearly visible DNA was spooled on to a glass rod and ethanol drained well. The DNA was resuspended in 100µlTE-buffer per milliliter of Lysis buffer used initially and stored at 4°C.

Lysis Buffer
100mM Tris HCl (Merck), pH8.5, 50mM EDTA (Sigma), pH8, 100mM NaCl (Merck), 0.5% (w/v) SDS (Merck)

b) Restriction Enzyme Digest of Genomic DNA:
The DNA concentration was measured by determining the light absorption at 260nm (OD260= 1µg/ml). 10µg were digested in a 50µl reaction volume at 37°C overnight. The digested DNA was mixed with 100µl TE buffer, extracted with phenol:chloroform:isomamylalcohol (50:49:1) and precipitated with $^1/_{10}$ volume 3M NaOAc and 2 volumes of 100% ethanol. The pellet was washed with 70% (v/v) ethanol and resuspended in 15µl of TE-Buffer. This sample was then analysed by Southern blot.

2.1.6 Digestion of DNA with restriction endonucleases

DNA was restricted according to the manufacturers specifications. The three most frequently occuring reaction conditions are listed below. Whereever possible restriction buffer supplied by the enzyme manufacturer was used. Restriction enzymes were obtained from: New England Biolabs, Boehringer Mannheim and Promega.

	Low salt	Medium salt	High salt
NaCl	0	50 mM	100 mM
Tris-HCl [pH 7.4]	10 mM	10 mM	50 mM
$MgCl_2$	10 mM	10 mM	10 mM
DTT	1 mM	1 mM	1 mM

2.1.7 Electrophoresis of DNA fragments

DNA molecules were fractionated according to size by elctrophoresis through an agarose gel. 1% (w/v) gels were generally used although 2% (w/v) gels were employed for analysis of DNA fragments less than 500 bp in size, and 0.7% (w/v) gels for DNA larger than 10 Kb. The appropriate volume of 1% (w/v) agarose (Gibco) in 1xTAE Buffer was made and heated to dissolve the agarose. The agarose solution was cooled to 60°C and ethidium bromide added to a concentration of 1µg/ml and poured into the gel cast. 1/10 volume Loading Buffer was added to samples and horizontal electrophoresis performed in 1xTAE Buffer at 5V/cm against standard molecular weight markers (Gibco-BRL).

50x TAE Buffer
0.2M Tris-Acetate (Merck), 50mM EDTA (Sigma)

5x Loading Buffer
0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue, 15% (w/v) Ficoll type 400 (Pharmacia) in ddH_2O

2.1.8 DNA elution using glass bead suspension

Purification of DNA was performed using the Gene Clean/Bio 101 kit and reagents. Briefly, after seperating the DNA on an agarose gel, the desired band was excised under long wavelength UV light and cut into small pieces. If the running buffer for the gel was TAE, 3 volumes of NaI Stock Solution (provided by manufacturer) were added to the gel slices. Alternatively, if the running buffer was TBE, 0.5 volumes of TBE Modifier (provided by manufacturer) and 4.5 volumes of NaI Stock Solution were added. The agarose was dissolved at 55°C. The agarose/DNA suspension was mixed with Glassmilk Suspension using 5µl/5µg total DNA. The suspension was incubated for 5 min. on ice and gently mixed every 1-2 min. The sample was twice centrifuged for 5 seconds at 13000 rpm and each time the supernatant was discarded carefully. The pellet was washed with 200µl NaI and incubated for 2 min. at 55°C to remove undissolved agarose. After spinning

for 5 seconds at 13000 rpm and removing the remaining supernatant, the pellet was washed twice with 10-50 volumes NEW Wash buffer and resuspended in 1 volume TE Buffer. The DNA was incubated for 3 min. at 55°C, spun down and the supernatant was recovered, which typically contained 80% of the DNA. This procedure was once repeated, recovering an additional 15% of DNA.

NEW Wash Buffer:
14 ml NEW Concentrate, 280 ml H_2O, 310 ml ethanol (100%)

TE Bufffer
10mM Tris HCl, pH7.5 (Merck), 1mM EDTA, pH8 (Sigma)

2.1.9 DNA ligation

T4 DNA ligase was used for all ligation reactions. This enzyme is suitable for sticky and blunt end ligations. The enzyme is unstable at temperatures higher than 30°C and is inhibited by high salt concentrations (>150mM).

The relative concentrations of vector DNA and fragment (insert) DNA used in the ligation reaction were calculated using the following formula: Fragment [µg/µl] = (MW Fragment/ MW Vector) x Vector [µg/µl]. To the appropriate amounts of Insert and Vector DNA 1µl 10x Ligase buffer, 1µl T4 Ligase [400 U/ml] and 10µl H_2O were added. Reactions containing small DNA fragments were incubated for 3-4 hours at room temperature. Ligation of large inserts required incubation overnight at 15°C to allow an annealing of sticky ends. Blunt end ligations were carried out in the presence of PEG 8000. The ligation reaction was stopped by incubation of the reaction mixture for 5 min. at 70°C.

10x Ligase Buffer for sticky end Ligation:
0.5M Tris-HCl, pH 7.5 (Merck), 0.1M $MgCl_2$ (Merck), 0.2M DTT (Sigma), 10mM ATP (Promega)

5x Ligase buffer for blunt end Ligation:
25mM Tris-HCl, pH 7.5, (Merck) 50mM $MgCl_2$ (Merck), 25% (w/v) PEG 8000 (Merck) 5mM ATP (Promega), 5mM DTT (Sigma)

2.1.10 Phosphatase treatment of DNA terminii

To prevent self ligation of digested vector in a ligation reaction, the vector was first was dephosphorylated. Briefly, the vector was digested with the appropriate restriction enzyme, extracted with chloroform, ethanol

precipitated and resuspended in medium salt restriction enzyme buffer (see 2.1.6). To this was added 0.2 units of calf intestinal alkaline phosphatase (Boehringer), SDS to a final concentration of 0.2% (w/v) and the sample incubated at 37°C for 30 min. The reaction was then terminated by the addition of EGTA to a final concentration of 20 mM to chelate all Mg^{2+} ions and heat inactivation of enzyme at 68°C for 15 min. The DNA was then phenol/chloroform extracted twice and ethanol precipitated.

2.1.11 Preparation of competent bacteria

A single bacterial colony was picked from a non-selective agar plate and innoculated in 5ml L-Broth. The culture was shaken at 37°C overnight. To 5ml overnight-culture 100 ml fresh L-Broth was added and incubated in a 500 ml flask at 37°C to an OD_{550} of 0.48. The mixture was cooled rapidly on ice and centrifuged at 2500 rpm for 5 min. at 4°C. The bacteria pellet was resuspended in 30ml ice cold TFB1. After incubation on ice for 15min. the suspension was pelleted at 2500 rpm for 5 min. at 4°C. The pellet was gently resuspended in 4ml ice cold TFB2 and icubated for 15 min. on ice. After spinning at 2500 rpm for 5 min. at 4°C the bacteria pellet was resuspended and aliquoted into microcentrifuge tubes at a volume of 200µl. The aliquots were stored at -70°C. This procedure produced competent bacteria capable of achieving an average of more than $5x10^8$ transformant colonies/µg of tranformed plasmid DNA.

TFB1
100mM RbCl (Sigma), 50mM Mn-II-Cl_2 (Merck), 30mM KOAc (Sigma), 10mM $CaCl_2$ (Sigma), 15% (v/v) Glycerol (Merck)

TFB2
10mM RbCl (Sigma), 10mM MOPS, pH7 (20.9g free acid/100ml, add ca.3ml M NaOH) (Sigma), 75mM $CaCl_2$ (Sigma), 15% (v/v) Glycerol (Merck).

2.1.12 Transformation of E. coli

The DNA was added to competent cells, mixed by gentle pipetting and incubated on ice for 30 min. to allow the DNA to be absorbed onto the cells. The cells were then induced to take up the DNA by heat-shock at 42°C for 90 seconds, after which 0.5 ml of L-Broth (Gibco) was added and the cells incubated at 37°C with shaking for 30 min. The cells were then gently spread onto a pre-dried agar plate containing the relevant antibiotic selection and incubated at 37°C overnight.

2.1.13 Bacterial cracking

To quickly screen for the uptake of an insert into a vector following ligation and bacterial transformation, a small pellet of transformed bacteria was lysed, the plasmid DNA released and analysed on an agarose gel. Specifically, 5ml of L-Broth with the appropriate antibiotic were inoculated with a single bacterial colony. After shaking the solution overnight at 37°C, 200µl of this culture were transferred to an microcentrifuge tube. The culture was spun for 1 min. at 13000 rpm. The pellet was vortexed with 50µl Cracking Buffer. 50µl of PCIA were added and the mixture was vortexed again. To test for the presence of an insert (i.e. a larger plasmid) 10µl of the aqueous upper phase were loaded onto a low percentage agarose gel (0.6-0.7%) alongside a control containing only the original cloning vector. Clones containing an insert where used for further analysis.

Cracking-buffer:
100mM TRIS-HCl (Merck), pH 7.5, 100mM EDTA (Sigma), pH 8, 100mM NaCl (Merck), 5% (v/v) Glycerol (Merck), 0.04% (w/v) Bromphenol Blue (Bio-Rad), 0.1% (w/v) SDS (Merck)

2.1.14 PCR cDNA cloning

a) First Strand cDNA Synthesis (Reverse Transcription)
First strand cDNA was generated using the PTKII oligonucleotide (Wilks *et al.* 1989) and 20µg total RNA. The subsequent PCR reaction was performed with Cetus Taq polymerase, using the provided buffers and oligonucleotides PTKI and PTKII (1 µg each). Specfically, 2µl RT Hyb Buffer were mixed with 150 ng 3' oligonucleotide and were added together with 10µl oligonucleotide to 20µg ethanol-precipitated total RNA. The mixture was incubated for 2 hours at 40°C to allow annealing. The resulting RNA/oligonucleotide complex was

mixed with 5µl 0.5M Tris, pH 8.3, 5µl 0.1M $MgCl_2$, 1µl RNasin, 1µl 1M NaPyrophosphate(optional), 2µl 1M KCl (Merck), 1µl 0.1M DTT (Sigma), 10µl 2mM dNTP's (Promega), 1µl AMV RT (20u, Boehringer Mannheim) and 1µl MoMuLV RT (20u, Pharmacia). The reaction mix was completed to a total volume of 50µl with DEPC-H_2O, incubated for 2 hours at 40°C and stored at -20°C.

RT Hyb Buffer:
2M NaCl (Merck), 0.2M PIPES pH6.5 (Sigma), 5mM EDTA (Sigma)

2M PIPES: (100ml)
60.86 PIPES (very acidic) (Sigma), 80ml DEPC H_2O, 10ml 10M NaOH (Merck), complete to 100ml with DEPC H_2O

O.5M EDTA: (100ml)
18.61g Na_2EDTA $2H_2O$ (Sigma), 70ml DEPC H_2O, 5ml 10M NaOH (Merck), complete to 100ml with DEPC H_2O

0.5M Tris pH8.3: (500ml)
30.28g Tris base (Merck), 400ml DEPC H_2O, <15ml conc HCl (Merck), allow to cool before adjusting pH, complete with DEPC H_2O to 500ml

0.1M $MgCl_2$: (100ml)
2.03g $MgCl_2$ 6 H_2O (Merck), 80ml DEPC H_2O, complete with DEPC H_2O

RNasin Ribonuclease Inhibitor
20U/µl Recombinant RNasin (Promega)

dNTP's: (2mM and 10mM)
100mM dATP, dCTP, dGTP, dTTP 2mM: 1:50 DEPC H_2O (Cat.No. U1240, pH7, ddH_2O), 10mM: 1:10 DEPC H_2O, (Promega)

AMV Reverse transcriptase:
10U/µl Boehringer Mannheim, RNA-dep. DNA polymerase

MoMuLV Reverse transcriptase:
1000U Pharmacia, cloned, FPLCpure

b) Polymerase Chain Reaction (PCR Amplification)
To hydrolyse the RNA, 5µl first Strand cDNA (from a) were incubated with 2µl 1M NaOH for 10 min. at 50°C. The reaction was then neutralised with 2µl 1M HCl. The neutralized cDNA was mixed with the following components: 10µl 10x Taq Buffer, 10µl 10mM dNTP's, 2µl 10mg/ml Gelatin, 1µl 5' oligonucleotide (0.5-1µg), 1µl 3' oligonucleotide (0.5-1µg), 0.5µl Taq polymerase (2.5U Cetus, Perkin-Elmer), 66µl ddH_2O. The reaction was overlayed with 100µl mineral oil (Sigma).

Oligonucleotide Purification:
Oligonucleotides were supplied in NH_4Cl solution (ca. 3ml), (20-mer/6600 kD: 0.2µM=ca.1.32mg, 40mM= ca. 264µg optimal) and distributed in 6 microcentrifuge tubes (a 400µll), 1ml 100% ethanol and 50µl 3M NaOAc, pH5.2 were added and oligonucleotides were precipitate at -20°C overnight. Nucleotides were pelleted at 15000 rpm for 15 sec at 4°C and resuspended in 100µl DEPC water. The concentration was measured: 1 OD_{260} =20 µg/ml

500mM KCl (Merck), 100mM Tris-HCl, pH8 (Merck), 100mM NaCl (Merck), 0.1mM EDTA (Sigma), 1% (v/v) Triton X100 (Sigma)

c) PCR Cycle (30 cycles)
To amplify the cDNA the following series of cycles were run: 1.5 min. at 93°C, 2 min. at 45°C and 4 min. at 63°C for 30 cycles obtaining 210bp fragments. The PCR products were cloned into pBluescript and sequenced.

2.1.15 λ Phage library screening

a) Plating λ Phage Clones/Libraries:
A single colony of *E. coli* bacteria ("SURE" strain, Stratagene) was used to inoculate a small volume of Luria broth containing 10mM $MgSO_4$ and 0.1% (w/v) maltose. This culture was incubated overnight at 30°C. NZY agarose plates were prewarmed to 37°C and λ Phage eluents (from λ Phage library stocks or from λ Phage plugs; see below) were diluted to a final concentration of 1:1000 in SM buffer. 600µl bacteria culture were mixed gently at 37°C for 15 min. with 1µl of λ Phage dilutent to allow the phage to adhere to the bacterial cell surface. 6.5 ml Top Agar (liquified and cooled to 37°C) were added and the mixture was plated on a 15cm diameter petri dish. After the overlay has solidified, the plates were icubated overnight at 37°C and then transferred to 4°C for storage.

NZY Broth (1000ml)
85mM NaCl (Merck), 8mM $MgSO_4.7H_2O$ (MW 246.38, Merck), 5g Yeast Extract (Gibco), 10g NZ Amine/Casein Hydrolysate (Gibco), adjust to pH7.5 with NaOH

NZY Plates
15g Difco Agar (Gibco) per liter NZY Broth (1.5%), Gibco. Approx. 50-80ml per 15cm Ø dish

Top Agar
3.5g Difco Agar per 500ml NZY Broth (0.7%). Approx. 7ml per 15cm Ø dish.

SM Buffer
100mM NaCl (Merck), 16.6mM $MgSO_4$ (Merck), 50mM Tris-HCl, pH 7.5 (Merck), 0.01% (w/v) Gelatin (Sigma)

LB Broth (1000ml):
10g NaCl, 10g Bacto-Tryptone, 5g Yeast Extract, H_2O to 1000ml final volume

Note: To harvest an amplified library from a 15cm Ø dish, 10ml of SM Buffer are spread on one plate containing ca. 50-80ml of NZY Agarose. This is allowed to diffuse overnight. The magnesium ion concentration of the eluent (library) is 8-16mM

b) Replica Filter Lifts:
Membranes/filters (Hybond N, Amersham) were labelled with a pencil (A: first, B: second lift). Three flat dishes were prepared containing: a) Denaturing Solution: 1.5M NaCl/ 0.5M NaOH, b) Renaturing Solution: 1.5M NaCl/ 0.5M Tris pH8 and c) 2x SSC.

For the first lift the membrane was gently lowered onto the plate and the position marked by piercing through the filter into the agar with a needle dipped into ink. The filters were allowed to adsorb for 2 min. before they were transferred to the Denaturing Solution (Plaque/DNA side down) and incubated for 2 min. Filters were then transferred to the Renaturing Solution for 5 min. followed by a washing step for 1 min. in 2xSSC. Filters were placed on Whatmann paper, "DNA/Plaque"-side up and allowed to dry.

20xSSC 3M NaCl (Merck), 0.3M sodium citrate (Merck), adjusted to pH7

c) Second Lift:
Membranes were placed on all plates at once and the membrane marked as described in (a) . Filters were allowed to adsorb for 10 min. and transferred to the Denaturing Solution, following the same Renaturing and washing procedure as described for the first lift. Filters were stacked with one interleaf, put between Whatmann paper and baked at 80°C in a vacuum for 2hours.

d) Hybridisation:
20ml of Hybridisation Solution (prewarmed to 42°C) were filled into a 15cm ∅ dish, before the filters were added one by one; each filter was submerged thoroughly. The prehybridisation was carried out at 42°Cfor 2-3 hours in a shaker. The prehybridisation buffer was drained and the filters were arranged as a rosette on the lid. The prehybridisation buffer was replaced by 15ml Hybridisation Solution containing the radiolabelled boiled DNA probe. Filters were added one by one and submerged thoroughly. The hybridisation was carried out at 42°C in 50% (v/v) formamide for 12-18hours in a shaker. The Hybridisation buffer was drained and filters were washed at room temperature in a series of buffers (10 min. per step): 2xSSC, 1xSSC, and 1xSSC/0.1% (w/v) SDS. A final wash was carried out in 1xSSC/0.1%SDS for 10 min. at 65°C. Filters were exposed to an auto radiography film and developed 12 to 18 hours later.

e) Isolating Plaques of Interest:
Signals on the auto radiogram were checked for the following criteria: is the signal present on both replicas and it must not align to an air bubble on the agar plate. Plaques of interest were cored with the wide end of a pasteur pipette and the plug was transferred into a microcentrifuge tube which contained 500µl SM Buffer and 20µl chloroform. The phage were eluted for

1-2hours at room temperature or at 4°C overnight. Such a λ phage stock can be stored for up to one year at 4°C.

f) In Vivo Excision of Phage Inserts Using λ ZAP System:
200µl of an overnight culture of XL1-Blue *E.coli* were mixed with 200µl Phage eluent from a plug and 1µl of R408 helper phage with a titer $>10^6$ pfu/ml. The mixture was incubated at 37°C for 15 min. followed by the addition of 3ml of 2xYT Media. This mixture was incubated at 37°C for 2-2.5 hours in a shaker. The tubes were heated at 70°C for 20min. and the suspension was spun at 2000 rpm (4000g) for 5min. The supernatant was decanted into a sterile tube. This supernatant contains pBluescript phagemid packaged as filamentous phage particles and can be stored at 4°C for 1-2 months.

50µl of this pBluescript phagemid stock was mixed with 200µl XL1-Blue overnight culture in a 1.5ml test tube. The same amount of XL1-Blue overnight culture was also mixed with 10µl $1:10^2$ pBluescript phagemid stock dilution and incubated at 37°C for 15 min. 100µl of each mixture were plated individually on 10cmØ LB/Ampicllin (50µg/ml) plates. The plates were pre-coated with 100µl IPTG [100mM] and 40µl X-Gal [2%] for blue/white selection. Plates were incubated overnight at 37°C. Plasmid DNA isolated from white colonies was purified and further analysed.

2.1.16 Generation of ExoIII nested deletions

a) Digest of Insert-bearing Plasmid DNA
Appropriate restriction enzymes were used to create a 3' overhang to protect sequencing primer sites and a 5' overhang which is susceptible to Exo-nuclease III digest. First, 30µg of purified, supercoiled plasmid DNA were digested to create the 3' overhang near the side which was to be protected from exo-nuclease attack. The digest was checked for completeness on an agarose mini gel. This plasmid DNA was then digested to create the 5' overhanging end. The double-digested DNA was extracted once with PCIA and once with CIA, then precipitated with 1/10 volume of 3M sodium acetate and 2 volumes of ethanol on dry ice for 15 min.

PCIA
Phenol (TE-10 saturated, pH8) (Fisons): Chloroform: Isoamylalcohol = 50:49:1 (Merck)

CIA
Chloroform : Iso-amylalcohol = 98 : 2 (Merck)

b) Exonuclease III/ S1 Nuclease Reaction:
The precipitated DNA was pelleted and dissolved in 60µl 1x ExonucleaseIII Buffer. Twelve microcentrifuge tubes each containing 45µl S1 Nuclease Reaction Mix were prepared. The DNA sample was incubated for 5 min. at 37°C and a 5µl aliquot was added to the first S1 Nuclease tube. To the remaining DNA, 150 units of Exonuclease III/picomole of 5' overhanging ends were added. The mixture was briefly vortexed and incubated at 37°C. At 30 second intervals 5µl DNA aliquots were transferred to individual S1 Nuclease tubes. The reactions were incubated for 30 min. at 30°C. Reactions were then transferred on to ice and 1µl aliquots from each time point analysed on an agarose gel to determine the extent of the deletions introduced. If the incubation times proved to be satisfactory, 50µl of TE-10 were added to each tube and the DNA was extracted once with PCIA and once with CIA. The DNA was then precipitated with 1/10 volume of 3M sodium acetate and 2 volumes of ethanol on dry ice for 15 min. After centrifugation for 15 min. at 15000 rpm the pellet was dried and dissolved in 10µl TE-10 buffer.

10x Exonuclease III Buffer:
0.66M TRIS, pH 8 (Merck), 66mM $MgCl_2$ (Sigma)

S1 Nuclease Reaction Mix:
530µl ddH_2O, 67µl 10x S1 Nuclease Buffer, 120Unitl S1 Nuclease (Pharmacia)

10x S1 Nuclease Buffer:
2M NaCl (Merck), 0.5M Sodium Acetate, pH 4.5 (Merck), 5% (v/v) Glycerol (Merck), 10mM $ZnSO_4$ (Merck)

c) Klenow Fill-in Reaction:
In order to allow ligation of the DNA, the chewed back DNA ends were blunted using the large fragment of DNA polymerase I. The following mixture was added to the individual DNA samples: 1µl 10x High Salt buffer, 1µl 0.5 mM dNTPs (Promega) and 1µl of Klenow fragment (2Units, Boehringer Mannheim). The mixtures were incubated at 37°C for 5 min.

10x High Salt Buffer:
100mM NaCl (Merck), 50mM Tris HCl, pH7.4 (Merck), 10mM $MgCl_2$ (Merck), 1mM DTT (Sigma)

d) Self Ligation and Transformation:
For each time point, the following was added to 3µl of the fill-in reaction: 5µl ddH_2O, 1µl 10x Ligation Buffer and 1µl T4 Ligase. The samples were incubated at 15°C overnight. To 10µl ligated DNA 50-200µl competent bacteria were added and incubated for 30-60 min. on ice. The mixture was transferred to a 37°C water bath for 2 min. and then plated on to agar plates containing a selective growth medium. The plates were incubated overnight

at 37°C and individual colonies were screened for the size of deletion introduced into the insert.

10x Ligation Buffer (New England Biolabs)
0.5M Tris-HCl, pH7.5, 0.1M $MgCl_2$, 0.2M DTT, 10mM rATP

2.1.17 DNA sequencing

Double-stranded DNA sequencing was performed using either the Sequenase 2.0 DNA Sequencing Kit (United States Biochemical) or the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

a) Sequenase 2.0 DNA Sequencing:
2-4µg of RNA-free plasmid DNA resuspended in 18µl H_2O were added to 2µl of 2M NaOH, 2mM EDTA and incubated for 5 min. at 37°C. The DNA was precipitated by adding 8µl 5M NH_4OAc, pH5 and 100µl 100% ethanol on dry ice for 5 min, pelleted at 4°C at 13000 rpm for 20 min, washed with 70% (v/v) ethanol, dried and resuspended in 7µl ddH_2O.

The DNA was mixed with 2µl Annealing Buffer and 1µl oligonucleotide primer (molar stoichiometry of primer to template= 1:1). The mix was incubated at 65°C for 2 min and allowed to cool slowly to 35°C. The sequencing reaction was carried out by mixing the annealed DNA sample with 1µl 0.1M DTT, 2µl 1x Labeling Mix, 1µl ^{35}S-dATP, and 2µl diluted Sequenase Polymerase (prediluted 1:8 in Enzyme Dilution Buffer). The reaction was incubated for 5 min. at room temperature.

From the labeling reaction, 3.5µl were added to each of the termination tubes containing 2.5µl of either of the four different ddNTPs: ddGTP, ddATP, ddTTP or ddCTP. After incubating for 5 min. at 37°C, the reaction was stopped by adding 4 µl Stop Solution. 2-3µl of each termination reaction were heat denatured and resolved on a 6% polyacrylamide/urea gel .The gel was prerun for 30 min. at 60W with 1xTBE as running buffer. DNA samples were run for 5 hrs at 6oW. The gel was fixed with 5% (v/v) methanol/ 5% (v/v) acetic acid and 15% (v/v) methanol for 15 min. and transferred on to filter paper. The gel was dried in a vaccum for 1 hr at 70°C and exposed to an auto radiogram overnight.

5x Annealing Buffer
200mM Tris-HCl, pH7.5, 100mM $MgCl_2$, 250mM NaCl

Enzyme Dilution Buffer
10mM Tris-HCl, pH7.5, 5mM DTT, 0.5mg/ml BSA

Labeling Mix:
7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP

Stop solution
95% Formamide, 20mM EDTA, 0.05% Bromphenol Blue, 0.05% Xylene Cyanol FF

Acrylamide/Urea gel (6%,):
8.3M Urea (Sigma), 20% (w/v) Acrylamide (Bio-Rad), 2.8 ml TBE (20%), 0.6 ml APS (10%) (Bio-Rad), 35 µl TEMED (Bio-Rad)

10x TBE buffer:
0.89 Tris HCl (Merck), 0.89M Boric Acid (Sigma), 2mM EDTA (Sigma)

b) Taq DyeDeoxy Terminator Cycle Sequencing:
Double-stranded DNA templates do not need to be denatured. 1µg of plasmid DNA in ddH_2O was mixed with: 3.2pmol oligonucleotide primer, 4µl 5x TACS Buffer, 1µl dNTP Mix, 1µl DyeDeoxy A Terminator, 1µl DyeDeoxy T Terminator, 1µl DyeDeoxy G Terminator, 1µl DyeDeoxy C Terminator and 0.5µl AmpliTaq DNA Polymerase in a total volume of 20µl. The reaction was overlayed with mineral oil (Sigma) and incubated for 25 cycles as follows: at 96°C for 30sec., at 50°C for 15sec. and at 60°C for 4min.. Samples were brought up to 80µl volume with ddH_2O and the aqueous phase transferred to a fresh microcentrifuge tube, followed by two extractions with 100µl of phenol:H2O:chloroform (68:18:14). Extension products were precipitated by adding 15µl of 2M sodium acetate, pH4.5 and 300µl 100% ethanol and centrifuging for 15min. at 13000 rpm at room temperature. Samples were washed once with 70% (v/v) ethanol, dried and resuspended in 4µl Formamide Buffer. Prior to loading onto Applied Biosystems 373A DNA sequencer, samples were heated at 90°C for two min..

5x TACS Buffer
400mM Tris HCl, 10mM $MgCl_2$, 100mM $(NH_4)_2SO_4$, pH9

dNTP Mix
750µM dITP, 150µM dATP, 150µM dTTP, 150µM dCTP

Formamide Buffer
80% Formamide, 10mM EDTA, pH8

2.1.18 Southern blot analysis

a) DNA transfer:
Purified DNA was digested using appropriate restriction enzymes and fractioned by electrophoresis in an agarose gel containing TAE buffer at 3 to 4V/cm. The gel was stained with ethidium bromide (5µg/ml) and photographed under longwave UV light. The DNA was then transferred to reinforced nitrocellulose (Schleicher & Schuell) by a modification of the Southern blot protocol(Sambrook *et al.* 1989). The agarose gel was incubated twice for 30min. in Solution A and then twice for 30min. in Solution B. The DNA

was transferred to nitrocellulose in Solution B over a period of 16-24hrs. The nitrocellulose filter was then allowed to dry and was baked in a vacuum at 80°C for 2 hours.

TAE Buffer
0.04M Tris-acetate (Merck), 2mM EDTA (Sigma)

Solution A
1.5M NaCl (Merck), 0.5M NaOH

Solution B
1M Ammonium Acetate (Sigma), 5mM NaOH

b) DNA detection

DNA was detected by hybridisation of a radiolabelled, complementary DNA probe. To denature purified DNA fragments, the samples were heated to 95°C for 5 min. and cooled on ice. For radiolabelling a labelling kit designed for random priming was used (Boehringer Mannheim) to incorporate [α^{32}P]dCTP (74MBq/ml, Amersham International) into DNA by adding: dATP, dTTP, dGTP (0.5nmol each), [α^{32}P]dCTP (0.37MBq), 2U Klenow DNA polymerase and 10% v/v of a solution containing a random mixture of hexanucleotides to the denatured DNA sample. The reaction was incubated for 30 min. at 37°C. The labelled DNA was purified from unincorporated nucleotides by using a chromatography column (Stratagen NucTrap). The activity of the probe was counted in 1µl fraction of purified DNA in a scintillation counter (Beckman Instruments).

The blot was pre-hybridised in a rotary hybridisation oven in Southern Hybridisation Buffer at 42°C overnight. Filters were washed once in 2xSSC/ 0.1%SDS at room temperature for 30 min, once in 1xSSC/ 0.1%SDS at room temperature for 30 min. and finally once in 0.1xSSC/ 0.1%SDS for 30min. at 60°C. The filters were then exposed to auto radiography film at -70°C for up to 7 days using intensifier screens, or to a phosphoimager storage plate up to 48 hours. Images were presented using computer supported graphics (Apple Macintosh, Microsoft Adobe Photoshop).

Southern Hybridisation Buffer
50% (v/v) Formamide (Fluka), 5xSSC, 1mg/ml Yeast tRNA (Sigma), 100µg/ml Heparin (Sigma), 1x Denhardt's Solution, 0.1% (v/v) Tween 20 (Sigma), 0.1% (w/v) CHAPS (Sigma), 0.5mM EDTA, pH 8 (Sigma)

20xSSC
3M NaCl (Merck), 0.3M sodium citrate (Merck), adjusted to pH7

50x Denhardt's Solution
1% (w/v) Ficoll (Pharmacia), 1% (w/v) polyvinylpyrrolidone (Pharmacia) and 1% (w/v) bovine serum albumin. (Sigma)

2.1.19 Genomic mapping of *Limk*1 using an interspecific backcross

The chromosomal localisation of *Limk*1 was determined by interspecific backcross analysis using a ([*Mus musculus* C57BL/6J x *M.spretus*]F1 x *M.spretus*) backcross (Rowe *et al.* 1994). 5µg of genomic backcross panel DNA (Jackson Laboratory, Maine) were digested in a 20µl volume, containing 10mM Tris-HCl, pH7.5, 50mM NaCl, 10mM $MgCl_2$, 1mM dithioerythritol and 40Units of *Bgl* II enzyme (New England Biolabs). The digested DNA was size fractionated on a 0.6% (w/v) agarose gel in TAE buffer (Sambrook *et al.* 1989). Gels were soaked twice for 30min. in 1.5M NaCl/ 0.5M NaOH, followed by two soaks in 1M ammonium acetate/ 20mM NaOH. DNA was then transferred in 1M ammonium acetate/ 20mM NaOH onto nylon-reinforced nitrocellulose (Schleicher &Schuell) and probed at high stringency with a ^{32}P-labeled *Eco*RI fragment derived from clone C5 (Sambrook *et al.* 1989). Auto radiography revealed a 2.2kb, 2.9kb, 3.3kb and 4.3kb band for the C57BL/6J genotype and a 2.9kb, 3.3kb and 6.5kb band for the *Mus spretus* genotype. The segregation of the 4.3kb C57BL/6J band and 6.5kb *M. spretus* band was then used to determine the segregation of the *Limk*1 locus in 94 backcross mice. Gene order was determined by minimizing the number of recombination events required to explain allele distribution patterns.

2.1.20 Total RNA isolation

a) Tissue culture:
Cells were grown to confluency and lysed directly in the culture dish with 1.8 ml GT Solution/ 100mm dish (ca 60cm^2) or 100µl GT Solution/ 10^6 cells of suspension culture.

b) Dissected tissue samples:
Tissue was transferred into ice cold Leibovitz Medium (L-15), washed with 1xPBS and centrifuged at 1200 rpm for 5 min.. The cell pellet was resuspended in 1.8 ml GT Solution and cells were triturated through a pipette.

c) RNA Purification:
The cell/ GT Solution mixture was transferred into a 4ml snap-cap tube and mixed thoroughly after adding 180 µl 2M NaAc, pH4, 1.8 ml phenol (H_2O saturated only for use with RNA) and 360µl Chloroform:Isoamyl Alcohol (CIA). The suspension was shaken well and cooled on ice for 15 min, before spinning at 10000 rpm for 20 min. at 4° C. The aqueous phase was transferred to a fresh tube and mixed with 1.8ml Isopropanol per 1.8ml RNA/GT Solution and left at -20°C overnight. The RNA was precipitated at 10000 rpm for 20 min. at 4°C and resuspended in 300µl GT Solution. 600µl 100% ethanol were added and the mixture was again incubated at

-20°overnight. The RNA was precipitated at 15000 rpm for 10 min. at 4°C, washed with 80% (v/v) ethanol, dried and resuspended in 50µl 0.2% (w/v) DEPC-SDS. This RNA preparation is ready to use for total RNA Northern blot or poly-A selection.

GT Solution
250g Guanidinium Thiocyanate (Fluka), 293ml ddH2O, 17.6ml 0.75M NaCitrate (Merck), pH7, 26.4ml 10% (w/v) Sarcosyl (Fluka). Compounds were dissolved at 65°C and prior to use 0.36ml 2-Mercaptoethanol were added per 50ml GT-Stock solution

2.1.21 Direct isolation of mRNA from animal tissue

a) Preparation of lysate from solid tissue:
Frozen animal tissue (20-50 mg) was ground in liquid nitrogen and the frozen powder was transferred to a homogenizer containing 1ml Lysis/Binding Buffer. The lysate was spun for 30-60 seconds to remove debris and combined with Dynabeads Oligo$(dT)_{25}$ which have been treated as described below (section b).

Lysis/Binding Buffer:
100mM Tris-HCl pH 8.0 (Merck), 500 mM LiCl (Sigma), 10mM EDTA pH8.0 (Sigma), 1% (w/v) LiDS Lauryl Sulfate) (Sigma), 5mM dithiothreitol (Sigma)

b) Conditoning of Dynabeads Oligo $(dT)_{25}$:
0.25ml Dynabeads (Dynal) were removed from the stock tube to a RNAse free microcentrifuge tube placed in a Dynal Magnetic Particle Concentrator (MPC). After 30 sec, when the suspension has become clear, the supernatant was removed and the beads washed with 0.2 ml Lysis/Binding Buffer. The supernantant was again removed using the Dynal MPC magnet. Washed Dynabeads were used immediately.

c) Direct mRNA Isolation from Crude Extract
The lysate from section a) was mixed with the prewashed Dynabeads Oligos $(dT)_{25}$ and incubated for 3-5 min. at room temperature to allow annealing. The tube was placed for 2 min. in the Dynal MPC magnet and the supernatant was removed. The pellet was washed three times with 0.5-1 ml Washing Buffer with LiDS and three times with 0.5 ml Washing Buffer. To elute mRNA, the pellet was mixed with 10-20µl 2mM EDTA, pH 8.0 and incubated for 2 min. at 65°C. The tube was placed in the Dynal MPC magnet to remove beads and the supernatant, containing the mRNA, was transferred to a fresh tube.

Washing Buffer with LiDS:
10mM Tris-HCl pH 8.0 (Merck), 0.15 M LiCl (Sigma), 1mM EDTA (Sigma), 0.1% (w/v) LiDS (Sigma)

Washing Buffer:
10 mM Tris-HCl pH 8.0 (Merck), 0.15 M LiCl (Sigma), 1mM EDTA (Sigma)

2.1.22 Northern blot analysis

Various organs were dissected from six week old C57BL/6J mice. RNA was extracted as previously described (Chomczynski and Sacchi 1987). 15µg of total RNA were heat denatured, size fractionated on a 1% (w/v) agarose/0.7% (v/v) formaldehyde gel in 1x MOPS Running Buffer. The fractionated RNA was then blotted onto Hybond-N membranes (Amersham) using 10xSSC transfer buffer. Membranes were probed at high stringency as described (Sambrook *et al.* 1989), using random primed ^{32}P-γ-dCTP labeled cDNA probes (Feinberg and Vogelstein 1983). Blots were first probed with a *Hinc*II/*Sma*I fragment of the amino-terminal half of *Limk*1, then with a cDNA probe for mouse glyceraldehyde phosphate dehydrogenase (GAPDH). Auto radiography was performed using a phosphoimager, and final images presented by computer based graphics (Macintosh, Adobe Photoshop).

10x MOPS
0.4M Na-Morpholinopropansulfate (MOPS, Sigma), 0.1M NaOAc (Sigma), 10mM EDTA (Sigma), adjust pH to 7.0 with NaOH (ca. 15ml/L)

2.1.23 SDS polyacrylamide gel electrophoresis

Gel equipment was assembled according to the manufacturers instructions. The resolving gel was prepared and filled to approx. twice the length of the comb teeth, from top edge of gel plate. Depending on the size of the protein to be analysed, different concentrations of polyacrylamide were used (7% (w/v) for ca. 170kD, 10% (w/v) for ca.80kD, 12-15% (w/v) for ca.40kD). The gel was immediately overlayed with H_2O-saturated butanol and allowed to set. The butanol was rinsed away with dH_2O and excess water removed with 3MM Whatman paper, before the stacking gel was poured on top of the resolving gel. The concentrations of the resolving gel were determined by the concentration of the stacking gel (3% (w/v) stacking gel for 5-8% (w/v) resolving.gel, 5% (w/v) stacking gel for 8-12% (w/v) resolving.gel and 6-8% (w/v) stacking gel for 15% (w/v) resolving.gel). The comb was inserted deep enough into the gel to exclude air and the gel was allowed to set. Gels were

transferred into gel clamps and inserted into the gel tank. Inner and outer chamber were filled with 1xSDS Running Buffer, the comb removed and wells rinsed with buffer. The protein samples were boiled for 5min. and put on ice. 5-15µl sample in 5x Sample buffer were pipetted into the wells and the gel was run at max. 200V for 40 to 50min. Gels were stained with Coomassie Blue for 10 min., destained with 40% (v/v) methanol, 10% (v/v) acetic acid for 45min. to overnight and dried on 2 layers of 3MM Whatman paper in a vacuum at max. 60°C for 45min.

Resolving Gel (10%)
For a total of 12 ml gel volume : 4.4 ml H_2O, 3 ml Acrylamid (40%, 29:1) Bio-Rad), 4.5 ml Tris-HCl pH 8.8, 60 µl SDS (20%), 60 µl APS (20%), 6µl TEMED (Bio-Rad).

Stacking Gel (5%)
For a total of 4 ml gel volume: 3.1 ml H2O, 0.38 ml Acrylamid (40%, 29:1), o.5 ml Tris-HCl pH 6.8, 20 µl SDS (20%), 20µl APS (20%), 4µl TEMED (Bio-Rad).

10x SDS Running Buffer (1000ml)
30 g Tris Base (Merck), 144g Glycine (Merck), 50 ml 20% (w/v) SDS pH8.3, 1-2 ml HCl

5x Sample Buffer
100mM Tris, pH8 (Merck), 4% (w/v) SDS (Merck), 50% (v/v) Glycerol (Merck), 50mM β-Mercaptoethanol (Sigma), 0.5% (w/v) Bromophenol Blue (Bio-Rad)

Comassie Blue:
10% (v/v) Acetic Acid (Merck), 0.3% (w/v) Coomassie R250 (Bio-Rad), 50% (v/v) Methanol

Fast Destain
10% (v/v) Acetic Acid, 40% (v/v) Methanol

Prestained MW-Markers (Sigma)

(Protein)	(Protein +Dye)	
180kD	-190kD	a2-Macroglobulin
116kD	-125kD	b-Galactosidase (E.coli)
84kD	-88kD	Fructose-6-Phosphate Kinase
58kD	-65kD	Pyruvate Kinase
48kD	-56kD	Fumarase
36.5kD	-38kD	Lactic Dehydrogenase
26.6kD	-27kD	Triosephosphate Isomerase

Preparation: 0.24g Urea, 500µl dH_2O, 200µl 5x Sample Buffer (Laemmli) were added to the marker vials, boiled and completed to 1ml, 20µl aliquots were made and stored at -20°C

2.1.24 Western blot analysis

Proteins separated by SDS-PAGE can be selectively identified using specific antisera by Western blotting. Specifically, he gel was immersed in Transfer Buffer for approximately 5 min. The gel was then placed on top of nitrocellulose paper (Hybond C, Amersham) presoaked in transfer buffer.

The gel/nitrocellulose was sandwiched between several layers of presoaked Whatman 3 MM and placed in the transfer apparatus (Biorad) with the nitrocellulose between the anode and the gel. The proteins were transferred from the gel onto the niotrocellulose by applying a current, which was dependent on the dimensions of the gel (mA=gel area cm^2 x 0.8). After 6 hrs the gel was then Coomassie stained to analyse whether transfer was complete.

The nylon membranes were blocked for 1-3 hours in Blocking Buffer. Membranes were then washed in TBST and incubated with the primary antibody, diluted in Blocking Buffer, for 2 hours at room temperature. After three washes in TBST, membranes were incubated for 1 hour at room temperature with the secondary antibody diluted in Blocking Buffer. Following three washes in TBST, the blot was developed using the Amersham ECL Chemiluminesence System, according to the manufacturers instructions.

Transfer Buffer
192 mM glycine, 25 mM Tris-HCl, pH 7.5, 0.1% (w/v) SDS, 20% (v/v) methanol

Blocking Buffer:
5%w/v Marvel milk powder, 0.9% (w/v) NaCl (Merck), 0.1% (v/v) Tween-20 (Sigma)
TBST buffer
Tris-buffered saline with 0.1% (v/v) Tween-20 (Sigma)

Primary Antibodies:
Anti-Glutathione-S-Transferase rabbit, polyclonal 1:1000 (gift from Dr. I. Gout)
Anti-phosphotyrosine, mouse monoclonal Py-20 (Sigma)

Secondary Antibodies:
Anti-mouse HRP 1:5000 or Anti-rabbit HRP 1:5000 (Southern Biotechnology)

2.1.25 Recombinant baculovirus culture

To express the kinase domain of Limk1 in baculovirus,the C-terminal half of Limk1 was cloned inframe into the *Eco* RI site of pAcG3X, a polyhedrin promoter driven, glutathione transferase containing expression vector suitable for generating recombinant Baculovirus (Davies *et al.* 1993).

a) General cell culture techniques and seeding densities for insect cells.
Spodoptera frugiperda (Sf9) cells were maintained as described in Summers and Smith (1987). Cells were routinely incubated at 27°C. The culture medium used throughout was IPL41 (Gibco) supplemented with 10% (v/v) FCS (Gibco). Routine culture was performed in the absence of antibiotics although 1% (v/v) fungizone (Gibco) and 0.1% (v/v) gentamycin (Gibco) were added for amplication of baculovirus stocks. Sf9 cells have a doubling

time of 18-24 hrs in IPL-41 containing 10% (v/v) FCS and thus needed to be subcultured at least twice a week. Confluent cells were routinely subcultured in a 75 cm^2 flask, by tapping the side of the flask gently against a surface, with minimal foaming, to dislodge cells. A fraction (1:4 to 1:20) of the cells were seeded into fresh flasks containing IPL-41. The flask was rocked gently to distribute the cells evenly and was then incubated at 27°C.

b) Transfection of Sf9 cells:
Plasmids containing a foreign gene were transferred to the AcNVP (polyhedron protein negative baculovirus) genome by recombination *in vivo*. 2 x 10^6 Sf9 cells were seeded into a 25 cm^2 flask and allowed to attach for 1 hr, after which the media was carefully aspirated and replaced with 0.75ml of Grace's medium (Gibco) supplemented with 1% (v/v) fungizone, 0.1% (v/v) gentamycin, 10% (v/v) FCS, and the flasks left at room temperature. The cells were washed twice in serum-free medium. The lipofectin was diluted 2:1 with sterile water and then mixed with an equal volume of a 1:2 mixture of the linear baculo DNA with the recombinant plasmid. This was left at room temperature for 15 min to allow the liposomes and the DNA to fuse, and then added to the cells in a minumum volume of serum free medium and left overnight. The following morning the transfection medium was replaced with complete medium and the recombinant virus harvested after 4-5 days.

c) Plaque Assay:
3.5 x10^6 viable Sf9 cells were seeded onto 60mm diameter culture plates in IPL41 containing 10% (v/v) FCS. The cells were allowed to attach for approximately 20 min while 10 fold dilutions of virus innoculum in 1 ml were prepared (10^{-1} to 10^{-7} for transfection mixes and 10^{-1} and 10^{-2} for virus picked plaques). Once the cells had attached to the surface of the dish the media was aspirated and the 1 ml diluted innoculum was gently added to each plate. The virus was evenly distributed by gentle rocking of the plate and then the plate incubated at 27°C for 1 hr. In addition a wild type virus and a uninfected control dish were set up. Meanwhile, a 1.5% (w/v) low melting point agarose (Gibco) overlay was prepared. For each 100 ml of final volume overlay required (4 ml per plate), 1.5 g of agarose was resuspended in 50 ml of water and autoclaved for 15-20 min. This was then combined with 50 ml of 2x Grace's Medium (Gibco) and the mixed solution equilibrated to 39°C in a waterbath. Following the 1 hr incubation period the virus was aspirated off from the cells and 4 ml of overlay agarose was carefully added to the edge of the dish so as not to disturb the cells. The dishes were then left undisturbed for at least 1 hr to allow the agarose to solidify, after which the dishes were placed into a sandwich box containing damp tissues, the box sealed and incubated for 7 days at 27°C. Plaques were examined 7 days after infection using phase contrast-L on a Nikon microscope (807344). The wild-type plaques (occlusion positive) contain polyhedra which are bead-like and very refractile to light, whereas the

recombinant plaques do not contain polyhedra and thus are light grey and less refractile (occlusion negative). The location of the occlusion negative plaques was marked on the bottom of the dish and the area of overlay picked with a pasteur pipette and subjected to further plaque purifications.

Baculogold DNA is linearised wild-type DNA that contains both a lethal deletion and a lac Z gene. Both are replaced after plasmid rescue by the foreign gene of the plasmid. Therefor only recombinant circular DNA will give rise to recombinants which can easily be recognised as colourless plaques on Xgal plates. Since linear DNA is unable to successfully transfect cells and generate virus particles, transfection using BaculoGold DNA results in almost 100% recombinant plaques.

d) Virus Amplification and Harvest ing of Infected Cells
For virus amplification, cells were allowed to attach in a 25 cm^2 flask, incubated with 0.5 ml of the original plaque purified virus and then the cells incubated for 4 days. The virus was harvested by centrifugation at 3000 g and then 1 ml used to infect cells in a 75 cm^2 flask. After harvesting 4 days post-infection, 1 ml of this virus stock was used to infect cells in a 150 cm^2 flask. Virus from this amplification was then used to build up a passage 5 virus stock which could then be titred for optimum protein production. To optimise protein production a number of infections were set up using varying dilutions of the virus stock in 25 cm^2 flasks, ie. from 1/10000 through to 1 to be harvested either 2 or 3 days post infection. Cells were harvested by gently tapping the flask to dislodge the cells. The media was then spun at 1000 g for 4 min, the supernatant decanted, the cells washed once in PBS and then again centrifugated. These cells were then lysed as described below.

2.1.26 Purification of GST-fusion protein expressed in baculovirus

Routinely, for large scale protein production, $4x10^7$ Sf9 cells in 30ml culture medium were infected with 1/100 volume of high titer virus stock and harvested on day three.Cells were washed in 1xPBS, lysed in 4ml Lysis Buffer. Debris was removed by brief centrifugation and the supernatant incubated for 30min. at 4°C with Glutathione-Sepharose beads (Pharmacia). The beads were washed three times with 50 volumes of Lysis Buffer and the GST-Limk1 fusion protein eluted in a minimum volume, dialysed and stored at -70°C.

Lysis Buffer:
50mM Tris-HCl (Merck), pH7.5, 50mM NaCl (Merck), 2mM PMSF (Sigma), 0.1mg/ml Leupeptin (Sigma), 0.1mM Na-Orthovanadate (Sigma), 1%Triton (Sigma)

Elution Buffer:
50mM Tris-HCl pH 7.5 (Merck), 150mM NaCl (Merck), 5mM Glutathione (GSH, reduced form, Sigma)

Dialysis Buffer:
50mM Tris-HCl pH 7.5 (Merck), 50mM NaCl (Merck), 1mM PMSF added fresh

2.1.27 Purification GST-fusion protein expressed in bacteria

a) Induction of GST-Fusion Protein Expression:
An overnight 50 ml bacterial culture was prepared in LB-Medium containing Ampicillin [50-100µg/ml] and diluted 1:10 into fresh, prewarmed LB medium. After 1 hr the culture was cooled to room temperature and mixed with 100-200µl 1M IPTG to a final concentration of 0.2-0.4mM. The culture was incubated for 3 hours at 27°C.

b) Purification of GST- Fusion Protein:
The bacterial suspension was pelleted at 3000 rpm for 20 min. and the pellet was resuspended in 6.25 ml Lysis buffer. The cell lysate was sonicated on ice for 2x1min. on setting 12 and centrifugated for 10 min. at 10000 rpm. The supernatant, representing the crude lysate was mixed with 50% (v/v) Bead Suspension and incubated for 30 min. at 4°C. After spinning for 5 min. at 1000 rpm the pellet was resuspended in 500µl Lysis Buffer and spun for 10 seconds at 13000 rpm. The supernatant was discarded and the pellet was washed twice with Lysis buffer. The purified GST-Fusion Protein, attached to the beads, can be used immediately for an *in vitro* kinase assay or can alternatively be eluted, by adding 0.5 ml Elution Buffer and 1mM PMSF to the beads. After repeating the elution process twice, the eluate was dialysed in 3 litres Dialysis Buffer overnight at 4°C. The purified and dialysed GTS-Fusion protein was flash frozen on dry ice and stored in small aliquots at -70°C.

Lysis Buffer:
50mM Tris-HCl, pH7.5 (Merck), 50mM NaCl (Merck), 2mM PMSF (Sigma), 0.1mg/ml Leupeptin (Sigma), 0.1mM Na-Orthovanadate (Sigma), 1% (v/v) Triton X-100 (Sigma)

Elution Buffer:
50mM Tris-HCl pH 7.5 (Merck), 150mM NaCl (Merck), 5mM Glutathione (GSH, reduced form, Sigma)

Dialysis Buffer:
50mM Tris-HCl pH 7.5 (Merck), 50mM NaCl (Merck), 1mM PMSF added fresh

2.1.28 *In vitro* kinase assay

Purified fusion protein bound to glutathione-beads was washed twice in kinase buffer and then incubated for 30 min. at 30°C in kinase buffer, with 1µl ^{32}P-αATP in the presence or absence of various divalent metal ions (5mM: Mn^{++}, Mg^{++}). The reaction was stopped with 1/5 volume 5x Sample Buffer. Phosphorylated proteins were then size fractionated by SDS-polyacrylamide gel electrophoresis, the gels fixed, dried and subsequently analysed by autoradiography (Sambrook *et al.* 1989).

1x Kinase Buffer:
50mM Hepes pH7.3 (Sigma), 150mM NaCl (Merck), 0.1% (v/v) Triton X-100 (Sigma), 5%v/v Glycerol (Merck), 0.1mM Sodium Orthovanadate (Sigma)

5x Sample Buffer:
100mM Tris pH8 (Merck), 4% (w/v) SDS (Merck), 50% (v/v) Glycerol (Merck), 50mM β-Mercaptoethanol (Sigma), 0.5% (w/v) Bromphenol Blue (Bio-Rad)

2.1.29 Phosphoamino acid analysis

Samples were prepared as described for *in vitro* kinase assay, the phoshorylated proteins fractionated by SDS-polyacrylamide gel electrophoresis and then transferred onto PVDF-membrane (Sambrook *et al.* 1989). Following autoradiography of the blot, radioactive bands were cut out from the membrane, and the protein eluted by acid hydrolysis (for 1hr at 110°C in 50µl constant boiling HCl). The sample was lyophylised and resuspended in 10µl pH 1.9 Buffer (2.2% (v/v) formic acid, 7.8% (v/v) acetic acid), which also contained 0.6µg each of phosphoserine, phosphothreonine and phosphotyrosine standards.

The sample was separated in two dimensions by thin-layer chromotography in a HTLE-7000 apparatus (CBS Scientific, CA, USA). After focusing the sample on precoated cellulose plates (Merck), elctrophoresis was performed in the first dimension using Runing Buffer 1, for 20 min at 1500 V. In the second dimension, Running Buffer 2 was used and 1300 V were applied for 20 min. After drying, the chromatography plate was then exposed to auto radiography film (Kodak XAR 5) at -70°C with intensifying screens. The position of the phosphoamino acid standards was identified by spraying 0.5% (w/v) ninhydrin in acetone on to the plates after which these were heated to 65°C for 20min.(Woodgett 1989).

Running Buffer (First dimension)
2.2% (v/v) formic acid, 7.8% (v/v) acetic acid, pH1.9

Running Buffer (Second dimension)
5% (v/v) acetic acid, 0.5% (w/v) pyridine, pH3.5

2.1.30 In situ hybridisation of frozen tissue sections

The tissue specific expression of Limk1 was studied using frozen sections of paraformaldehyde fixed mouse tissues. The *in situ* hybridisation protocol applied here is based on the detection of digoxigenin labelled RNA by alkaline phosphatase conjugated anti-DIG Fab-fragments (Boehringer Mannheim) as described (Wilkinson 1992; Birren *et al.* 1993).

a) Microscope Slide Preparation:
Microscope slides were either soaked overnight in Dichrol in a fume hood or for 1 hr in surface cleaning detergent (Decon 90). The slides were rinsed in ddH_2O and dried for 20min. at 150°C. Slides were then dipped for 20-60 seconds in a solution of 2% (w/v) TESTA (Sigma A-3648) in acetone, followed by two washing steps in acetone and three washing steps in DEPC-treated water. After drying the slides overnight, they can be stored dry, at room temperature. All glass ware should be baked at 180°C for twelve hours and then treated as RNAse-free. Double-distilled water (or water of equivalent quality) was made RNase-free by treating one liter of water with 1ml Di-Ethyl-Pyrocarbonate (DEPC, Sigma) for 12 hours at room temperature, followed by autoclaving at 120°C for 20 min. to remove remaining traces of DEPC.

b) Section Preparation:
Sections were prepared from mouse embryos of different ages by incubating of the tissue overnight in 4% (w/v) paraformaldehyde in DEPC-PBS at 4°C, followed by another incubation overnight at 4°C in 30% (w/v) Sucrose with 0.02% (w/v) Azide. Tissues were then dipped in Pre-OCT (OCT:30% (w/v) Sucrose =1:1) and embedded in OCT. Embedded tissues were stored at -20°C. 15-20µm sections where cut on a Bright Instrument Company cryostat and stored at -20°C until further processing.

Phosphate Buffered Saline (PBS)
140mM NaCl (Merck), 3mM KCl (Merck), 1mM K_2HPO_4 (Merck), 7mM $Na_2HPO_4 \cdot 2H_2O$ (Merck), 0.5mM $MgCl_2 \cdot 6H_2O$ (Merck)

4% (w/v) Paraformaldehyde in 1x PBS:
8g EM-grade Paraformaldehyde (Sigma) was dissolved in 200ml DEPC-H_2O and 200µl 5M NaOH by stirring for 30min. at 60°C. Two PBS tablets (Sigma P-4417) were added and allowed to dissolve. The solution was cooled to room temperature and the pH was adjusted to pH7 with 1M HCL.

c) Template Preparation:
To generate digoxigenin-labelled probes templated DNA was linearised to allow run-off transcription using the RNA polymerase promoter present upstream of the cloned insert (probe sequence). Specifically 20-40µg plasmid DNA were digested with appropriate restriction enzymes and precipitated in 1/9 volume RNAse-free 3M NaOAc, pH 4.8 and 2 volumes 100% ethanol at

-70°C for 30 min. The DNA was resuspended in 20µl DEPC-H_2O and diluted to a final concentration of 0.5µg/µl.

d) Preparation of Digoxigenin (DIG) Labelled RNA Probes:
In a final volume of 50µl the following reagents were added: 2.5µg template DNA, 10µl 5x Transcription Buffer (supplied by manufacturer), 5µl 100mM DTT, 20µl 2.5x rNTP/DIG-UTP Mix, 1µl RNasin (10U/µl) and RNA polymerase (T3, T7 or SP6; Boehringer Mannheim). The mixture was incubated for 2hrs at 37°C and a small aliquot (2µl) was retained. The rest was then incubated with 20U RNAse-free DNAse at 37°C for 10 min.

After the incubation another 2µl aliquot was removed. This and the previous aliquot were then analysed side-by-side on an agarose gel to test for complete degradation of the DNA template. To the remaining sample 52µl of STOP buffer (1% (w/v) SDS, 20mM EDTA, pH8) were added and the sample was purified using a Sephadex G-50 (Pharmacia) spin column. The column was prepared by packing the base of a 3ml syringe with siliconised, baked glass wool, and loaded with 5ml of G-50 slurry (DEPC-treated, autoclaved Sephadex in 0.3M NaOAC, pH6 and 1% (w/v) SDS). After spinning the syringe at 2000 rpm for 3 min. the buffer was poured off. The sample was loaded onto the column and centrifuged (2000 rpm/ 3min., tabletop centrifuge). The eluate was transferred to a fresh microcentrifuge tube and precipitated with 1/9 volume RNAse-free 3M sodium acetate, pH 4.8 and 2 volumes 100% ethanol at -70°C for 30 min. The DNA was washed with 95% (v/v) ethanol and dissolved in 50µl 40mM $NaHCO_3$/60mM Na_2CO_3. One microliter of the sample was used in a UV-spectrophotometer to determine the amount of single-stranded RNA probe generated (OD_{260nm} 1= 40µg/µl). To hydrolyse the RNA strands into smaller fragments which can diffuse into the tissue more readily, the sample was incubated at 60°C for various amounts of time (t) according to the following formula:

$$t = \frac{L_o\,[kb] - L_f\,[kb]}{0.11 \times L_o\,[kb] \times L_f[kb]}$$

Lo: original length of transcripts (= length of template DNA) in kb
Lf: desired length of probe fragments in kb (idealy 0.2-0.4kb)

To stop hydrolysis, the sample was precipitated with 5.5µl 1M sodium acetate, pH4.8 and 100µl 100% ethanol at -70°C for 30 min. The sample was washed with 95% (v/v) ethanol and suspended in 200µl Hybridisation Buffer to a final concentration of 40µg/ml. Probes were then stored at -70°C. The incorporation of DIG-rUTP into the RNA probes was assayed by dot-blot analysis: a Hybond-N nylon membrane (Amersham) was subdivided into small squares using a soft pencil. 1-5µl of probe were spotted on to the membrane and allowed to air dry. The RNA was then cross-linked to the membrane using UV light (Stratagene UV-Crosslinker, 1200µJoules). All subsequent treatments were carried out on a shaking platform and at room

temperature: 1min. in Buffer 1, 30 min. in Buffer 2 (blocking), 1min. in Buffer 1, 30min. To detect the DIG-groups incorporated into the probe, the membrane was incubated in a 1:5000 dilution of anti-DIG antibody coupled to alkaline phosphatase (Boehringer Mann.) in Buffer 1 for 30min. The membrane was then washed twice for 15min. in Buffer 1 and for 2min. in Buffer 3. The colour reaction was carried out for 5-20min. in Colour Substrate Buffer. The reaction was stopped with Buffer 4 and the membrane stored moist, wrapped in cling film.

2.5x rNTP/DIG-UTP Mix
2.5mM rATP, 2.5mM rCTP, 2.5mM rUTP, 2.5mM rGTP (Promega), 0.87mM DIG-rUTP (Boehringer Mannheim)
Hybridisation Buffer
50% (v/v) Formamide (Fluka), 1mg/ml Yeast tRNA (Sigma), 100µg/ml Heparin, 5xSSC, 1x Denhardt's Solution, 0.1% (v/v) Tween 20 (Sigma), 0.1% (w/v) CHAPS (Sigma), 0.5mM EDTA pH8 (Sigma).

20xSSC
3M NaCl (Merck), 0.3M sodium citrate (Merck), adjusted to pH7

50x Denhardt's Solution
1% (w/v) Ficoll (Pharmacia), 1% (w/v) polyvinylpyrrolidone (Pharmacia) and 1% (w/v) bovine serum albumin. (Sigma).

Buffer 1
100mM Tris-HCl, pH7.5 (Merck), 150mM NaCl (Merck)

Buffer 2
100mM Tris-HCl, pH7.5 (Merck), 150mM NaCl (Merck), 5% w/v Blocking Reagent (Marvel)

Buffer 3
100mM Tris-HCl, pH7.5 (Merck), 100mM NaCl (Merck), 50mM $MgCl_2$ (Merck)

Buffer 4
10mM Tris-HCl, pH7.5 (Merck), 10mM EDTA, pH8 (Sigma)

Colour Substrate Buffer
Buffer 3 + 0.45% v/v Nitrobluetetrazolium (in 70% dimethyl formamide) and 0.35% v/v 5-bromo-4-chloro-indoyl phosphate (in 100% DMF) (both from Boehringer Mannheim)

e) Fixation and Hybridisation of Tissue Sections
The slides were warmed to room temperature and dried at 50°C for 15 min. The microscope slides were then placed in suitable slide racks. All following treatments were carried out in baked, glass troughs and at room temperature unless otherwise indicated.

After drying, the sections were fixed for 20min. in 4% (w/v) paraforaldehyde, pH7 (Sigma), then washed twice for 5min. in DEPC-PBS. To allow better penetration of the probe, the samples were then permeabilised in 50µg/ml Porteinase K (Boehringer Mannheim) in Protease Buffer, then rinsed once in DEPC-PBS. To reduce background signal, the tissue sections were then acetylated. This was done by placing them in 200ml 0.1M Triethanolamine-HCl, pH8 and slowly adding 420µl acetic anhydride. The

samples were allowed to stand for 10min. and rinsed for 5min. in DEPC-PBS and 5min. in 0.85% (w/v) sodium chloride. A subsequent alcohol series to dehydrate the slides is optional (30, 50, 70. 80, 90, 100% (v/v) ethanol, 3min. each). The tissue sections were now ready to be hybridised. All hybridisations were carried out at 60°C. The microscope slides were covered with 25x55mm siliconised coverslips and kept in a moist chamber. For the prehybridisation (3-4hrs) 250µl Hybridisation Buffer per microscope slide were used. The probe was used in a final concentration range of 1-4µg/ml.

Protease Buffer
50mM Tris-HCl, pH7.5 (Merck), 5mM EDTA, pH8 (Sigma)

Hybridisation Buffer
50% (v/v) Formamide (Fluka), 1mg/ml Yeast tRNA (Sigma), 100µg/ml Heparin (Sigma), 5xSSC, 1x Denhardt's Solution (Sigma), 0.1% (v/v) Tween-20 (Sigma), 0.1% (w/v) CHAPS (Sigma) 0.5mM EDTA pH8 (Sigma).

f) Washing Steps after Hybridisation:
After hybridisation it was no longer necessary to use RNase-free solutions, but it was important not to let the sections dry out. The hybridisation buffer containing the probe can be collected and reused several times. The slides were washed as followed: 10min./ 60°C in Buffer A, 10min./ 60°C in Buffer B, and twice for 20min./ 37°C in Buffer C. This was followed by an RNase treatment to further reduce unspecific background signal by incubating the slides for 30min./ 37°C in Buffer C containing 20µg/ml RNase A and 10U/ml RNase T1. Slides were washed for 10min. at room temperature in Buffer C, for 30min./ 60°C in Buffer D, two times for 10min./ 60°C in Buffer E, for 10min./ room temperature in PTw and finally 15min. in PBT.

Buffer A	1xSSC, 0.3% (w/v) CHAPS (Sigma)
Buffer B	1.5xSSC, 0.3% (w/v) CHAPS (Sigma)
Buffer C	2xSSC, 0.3% (w/v) CHAPS (Sigma)
Buffer D	0.2xSSC, 0.3% (w/v) CHAPS (Sigma)
Buffer E	0.3% (w/v) CHAPS (Sigma), 0.1% (v/v) Tween-20 in PBS
PTw	0.1% (v/v) Tween-20 (Sigma) in PBS
PBT	2mg/ml BSA (Fraction V, Sigma), 0.1% (v/v) Triton X-100 (Sigma) in PBS

g) Anti DIG Antibody Detection:
Slides were treated for 1 hr with PBT + 20% (v/v) heat inactivated newborn sheep serum (NSS). This treatment was followed by an overnight incubation at 4°C in 1/1000 preabsorbed Anti-DIG in PBT + 20% (v/v) NSS. An amount of 250µl solution was used per slide. The slides were covered with siliconized coverslips. Slides were washed three times in PBT at room temperature for 30 min. following one wash in alkaline phosphate (AP) buffer for 5 min. and one wash in AP buffer + 5mM Levamisole (Sigma) for 5 min. at room temperature. To develop the colour reaction, slides were incubated with fresh Colour Substrate Solution, using 250µl solution/slide.

Slides were not covered with coverslips. After 2-20hrs (depending on the abundance of mRNA) slides were washed for 5 min. in PBS at room temperature. Before mounting, the slides with glycerol they were incubated for 15min. to 2hrs in MEMFA.

Preabsorbtion of Anti-DIG Antibody:
The generation of 1ml of 1:200 Anti-DIG antibody required 8-10 rat embryos aged 12.5 days. Embryos were fixed for 2hrs at room temperature in 4% (w/v) paraformaldehyde, washed for 5 min. in DEPC-PBS and stored at -20°C in Methanol. To rehydrate, the embryos were washed for 5min. at room temperature in a descending Methanol series (75%, 50%, 25% (v/v) in PTw). After a final wash for 5 min. in PTw embryos were transferred to 10%NSS in PTw. Embryos were then dissociated using a 5ml pipette, followed by a pasteur pipette. The tissue was incubated for 1hr at room temperature on a rotating wheel and centrifuged at 3000 rpm for 5 min. An equal volume of an anti-DIG antibody diluted 1:200 in 1%NSS/PTw was added. This mixture was incubated for 3hrs at room temperature on a rotating wheel, before spinning at 3000 rpm for 5 min. The pellet was dicarded and the antibody-containing supernatant brought to a final dilution of 1:1000 in 20%NSS/PBT.

Alkaline Phosphatase Buffer:
100mM Tris-HCl, pH 9.5 (Merck), 50mM $MgCl_2$ (Merck), 100mM NaCl (Merck), 0.1% (v/v) Tween 20 (Sigma)

MEMFA:
1x MEMfa Salts, 3.7% (v/v) Formaldehyde (Fluka). Made up fresh each time.

5x MEMfa Salts:
0.1M MOPS (free acid, Sigma), 2mM EGTA (Sigma), 1mM $MgSO_4$ (Sigma), adjust pH to 7.5 with NaOH

Colour Substrate Buffer
Buffer 3 + 0.45% v/v Nitrobluetetrazolium (in 70% dimethyl formamide) and 0.35% v/v 5-bromo-4-chloro-indoyl phosphate (in 100% DMF) (both from Boehringer Mannheim)

2.1.31 Whole mount in situ hybridisation

a) Specimen Fixation:
Embryos of different ages (E6.5 to E9.5) were dissected in DEPC-PBS by removing the yolk sack and the amnion. The embyros were first briefly incubated at room temperature, then for 1 hr at 4°C in 4% (w/v) paraformaldehyde in DEPC-PBS. The embryos were washed for 1 hr in PTw at 4°C by gently rocking. At this point the embryos can be stored for several weeks at -20°C, when transferred to 100% methanol.

b) Specimen Bleaching:
To reduce background signal substantially the embryo tissue was treated for 2-3 hours with a mixture of 100% methanol/30% (v/v) hydrogen peroxide and subsequently washed several times with 100% methanol.

c) Template Preparation
See section 2.1.25 (c).

d) Preparation of DIG labelled Probe:
See section 2.1.25 (d).

e) Fixation and Hybridisation of Whole Mount Specimens:
Embryo tissue was fixed in 4% (w/v) paraformaldehyde, pH7 (Sigma), then washed twice for 5min. in DEPC-PBS. To allow better penetration of the probe, the samples were then permeabilised in 20µg/ml Proteinase K (Boehringer Mannheim) in Protease Buffer, then rinsed once in DEPC-PBS. A subsequent alcohol series to dehydrate the tissue was performed: 75%, 50%, 25% (v/v) Methanol in PTw, 5min. each, followed by twice PTw for 5 min. Embryos were then treated for 5 min. with 20µg/ml Proteinase K followed by 2mg/ml Glycine for 5 min. Tissue was washed twice in PTw, before incubating in 0.2% (v/v) Glutaraldehyde/ 4% (w/v) Paraformaldehyde for 20 min. Embryos were washed again three times with PTw before adding 0.1% (v/v) sodium borohydride in PTw. The tissue was incubated for 20 min. and then washed three times with PTw. The embryos were transferred to microcentrifuge tubes and were ready to be hybridised. All hybridisations were carried out at 63°C. For prehybridisation (1hr) 250µl Hybridisation Buffer per microcentrifuge tube were used. The probe was used in a final concentration range of 1-4µg/ml.

PTw:
1xPBS (Sigma), 0.1% (v/v) Tween-20 (Sigma)

10xPE
100mM PIPEs pH 6.8 (Sigma), 10mM EDTA pH 8 (Sigma)

Protease Buffer
50mM Tris-HCl, pH7.5 (Merck), 5mM EDTA, pH8 (Sigma)

Hybridisation Buffer
50% (v/v) Formamide (Fluka), 100µg/ml Yeast tRNA (Sigma), 0.05mg/ml Heparin (Sigma), 0.1% (w/v) BSA (Sigma), 1% (w/v) SDS (Merck), 0.75M NaCl (Merck), 1x PE

f) Washing Steps after Hybridisation:
The embryos were washed as follows: twice for 30min./ 63°C in Wash 1, twice for 30min./ 50°C in Wash 1.5, and for 2min./ 37°C in RNAse Buffer. This is followed by an RNase treatment to further reduce unspecific background signal: 60min./ 37°C in RNAse Buffer containing 100µg/ml RNase A (Boehringer Mannheim) and 100U/ml RNase T1 (Boehringer

Mannheim). The tissue was washed for 30min./ 50°C in Wash 2, for 30min./ 50°C in Wash 3 and two times for 30min./ 50°C in Wash 4. Samples were then incubated for 20 min. at 70°C in a heating block.

Was h 1 300mM NaCl (Merck), 1xPE, 1% (w/v) SDS (Merck)
Was h 1.5 50mM NaCl, 1xPE, 0.1% (w/v) SDS
Was h 2 300mM NaCl, 50% (v/v) Formamide (Fluka),1xPE
Was h 3 150mM NaCl, 50% (v/v) Formamide,1xPE, 0.1% (v/v) Tween (Sigma)
Was h 4 500mM NaCl, 1xPE, 0.1% (v/v) Tween

RNAse Buffer
500 mM NaCl, 10mM PIPES pH 7.2 (Sigma), 0.1% (v/v) Tween-20
PBT
2mg/ml BSA (Fraction V, Sigma), 0.1% (v/v) Triton X-100 (Sigma) in PBS

g) Anti DIG Antibody Detection:
Embryos were treated for 1 hr with TBST + 10% (v/v) heat inactivated newborn sheep serum (NSS) + 2mM Levamisole. This treatment was followed by an overnight incubation at 4°C in 1/2000 preabsorbed Anti-DIG in TBST + 1% (v/v) NSS + 2mM Levamisole. Embryos were washed six times in TBST at room temperature for 15 min. following three washes in TBST for 1hr and two washes in AP buffer + 5mM Levamisole (Sigma) for 30 min. at room temperature. To develop the colour reaction embryos were incubated with fresh Colour Substrate Solution. After 2-20hrs embryos were washed three times for 5 min. in PET at room temperature to stop the colour reaction. Embryos were then washed three times in TBST for 5 min, followed by several dehydration steps: samples were washed for 5 min. in an ascending series of ethanol (30%, 50%, 70%, 100%) followed by washes for 5 min. in a descending series of ethanol (70%, 50%, 30) to rehydrate the tissue. Samples were washed for 5 min. in TBST and incubated for 1hr in 50% (v/v) GPET and 1hr in 75% (v/v) GPET. Embryos were mounted with glycerol and examined under a stereo microscope.

Preabsorbtion of Anti-DIG Antibody:
prepared as described in 2.1.25

TBST:
140mM NaCl (Merck), 2.7mM KCl (Merck), 25mM Tris-HCl pH 7.5 (Merck), 0.1% (v/v) Tween-20 (Sigma)

Alkaline Phosphatase Buffer:
100mM Tris-HCl, pH 9.5, 50mM $MgCl_2$ (Merck), 100mM NaCl, 0.1% (v/v) Tween 20

PET buffer
1xPBS, 0.5mM EDTA pH 8 (Sigma), 0.1% (v/v) Tween-20

50% GPET:
1:1 mixture of Glycerol (Merck) and PET buffer

2.2. Materials

2.2.1 Molecular biology supplies

a) Growth Media and Solutions:
Where buffers and solutions have not been listed with the appropriate method in section 2.1., they were prepared as previously described by Sambrook et al., 1989. Reagents were always molecular biology grade and were tested by the manufacturer for the presence of contaminating nucleases.

Water used was always deionised and filtered using a Millipore water-purification system (to 15-18 megaOhm resistance) and subsequently auto-claved (ddH_2O). For use in experiments which involve intact RNA, water was di-ethyl-pyro-carbonate treated before being autoclaved .

Growth media (Luria Broth, NZYCM Media for lamda phage growth), antibiotics and agarose were purchased from Gibco-BRL.

b) Other Materials:
Plastic labware was washed, autoclaved and dried, while glassware was washed and baked at 180°C for 2-4 hours. Pipettes, micropipette tips and microcentrifuge tubes were supplied by different manufacturers (Costar, Treff, Multi, Gilson).

Auto radiography film was supplied by Kodak (XR-2) and Fujichrome. Images were presented using computer supported graphics (ImageOuant Phosphoimager, Apple Macintosh, Microsoft Adobe Photoshop).

2.2.2 Tissue culture supplies

a) Culture Media and Animal Sera:
All culture media (DMEM, Leibovitz L-15 Medium, IPL-41, 2x Grace's Insect Cell Medium), animal sera, lipofectin, glutamine, gentamycin, yeastolate and fungizone were obtained from Gibco-BRL. Tissue culture grade water was supplied by Imperial Laboratories.

b) Other Reagents:
Trypsin solution used for passaging mammalian cells was made up as a 3mg/ml (30,000 units/ml) solution of bovine pancreatic trypsin (Sigma) in DMEM-Calcium and Magnesium free medium (kindly supplied by the Imperial Cancer Research Fund).

The trypsin activity was neutralised using SBTI-DNAse solution. This solution consists of 0.52 mg/ml soybean trypsin inhibitor, 0.04 mg/ml bovine

pancreatic DNAse and 3mg/ml BSA Fraction V (all from Sigma) made up in DMEM.

Type IV collagen (Sigma), used to cultivate PC12 cells, was used at 30µg/ml in tissue cultiure grade water.

c) Disposable Plastic Ware

Tissue culture flasks, Petri dishes, multiwell trays, cell scrapers, syringes and centrifugation tubes were supplied by Falcon or Nunclon Delta (GIBCO). Microscope slides and coverslips were supplied by BDH/Merck.

2.2.3 Animals

Adult and embryonic C57Bl/6 mice were obtained from the Biological Services Unit, University College London. The age of embryonic animals was estimated by the presence of a vaginal plug post coitum and confirmed within a twelve hour range on the basis of crown-rump length and the number of somites.

Genomic DNA from *Mus spretus* and ((*M.musculus* x *M.spretus*) x *M.spretus*) offspring was supplied by The Jackson Laboratory, Bar Harbour, Maine/U.S.A.

3

Results

3.1 Cloning and sequence of murine *Limk1* cDNA

Degenerate primers were used in a modified reverse transcriptase-PCR based approach to initially isolate a 210bp cDNA fragment encoding part of the catalytic kinase domain of Limk1 (Wilks *et al.* 1989; Reith *et al.* 1990). Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out on mRNA isolated from a conditionally immortalised, embryonic day 13 mouse liver cell line, tsA58-L25, which exhibited an intermediate phenoytpe between the hepatocyte and bile ductular cell lineages (to be published elsewhere). The sequence of the degenerate oligonucleotides used as primers (PTK1 and PTK2) was based on the conserved motifs VI and IX found in the catalytic domain of protein kinases (Hanks *et al.* 1988; Hunter 1991). Because of the conserved structure of protein kinase domains, the predicted size of the cDNA fragments obtained was 210bp. These fragments were cloned into a plasmid vector, sequenced and selected for the presence of protein kinase domain-like sequences. Fifty clones were screened, and contained fragments of the following sequences: Insulin receptor, hyk, Jak1, Jak2, ufo, flk1 and flt. Two encoded the same subdomain fragment of a novel protein kinase, which we have subsequently named *Limk1*.

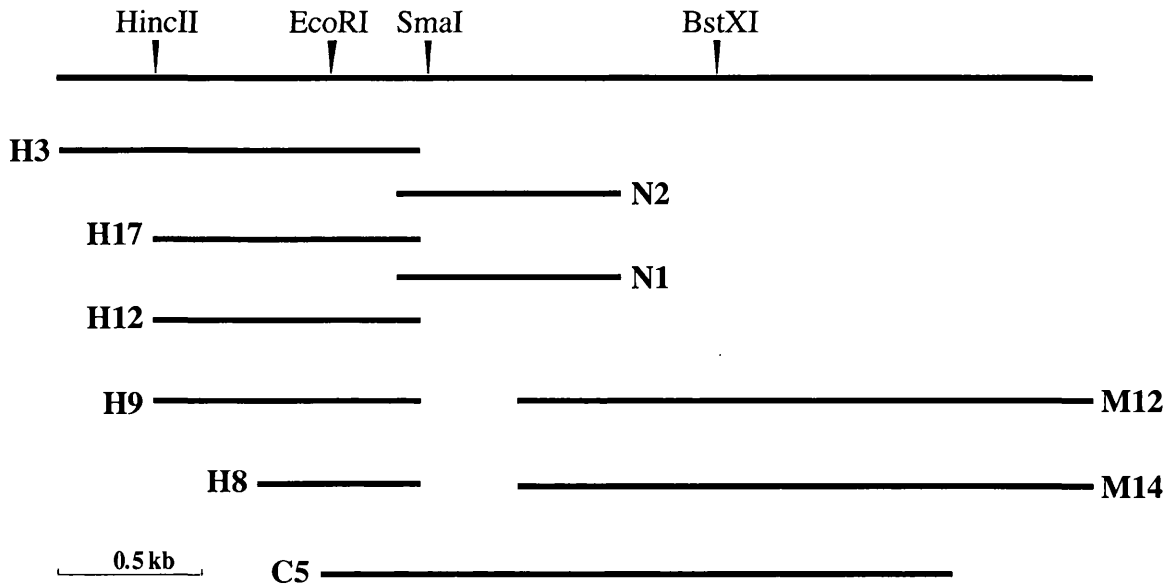


Figure 5: Cloning of *Limk1* cDNA. The alignment of *Limk1* cDNA clones used to construct the composite cDNA sequence is shown. Clones C5, H3, H8, H9, H12, H17, M12 and M14 were isolated from adult pancreas (C), adult spinal cord (H clones) and fifteen day whole embryo (M clones) cDNA libraries. Clones N1 and 2 were generated by reverse-transcriptase polymerase chain reaction on adult spinal cord mRNA.

To obtain full-length cDNA clones of *Limk1*, the 210bp cDNA fragment was used to probe cDNA libraries from different tissues. PCR analysis using primers derived from the sequence of the 210bp *Limk1* fragment identified the presence of *Limk1* like sequences in poly(dT) primed adult mouse pancreas and brain cDNA libraries. When screening the pancreas cDNA library, one clone (C5) was isolated which encoded 2kb of cDNA wih a single large open reading frame (Figure 5). However, clone C5 lacked a poly(A)$^+$ tail and the open reading frame was truncated at the 5' end. To isolate additional 5' sequence, a *Limk1* specific bookshelf cDNA library was generated from adult mouse spinal cord mRNA using a primer which mapped near the 5' end of clone C5. Screening this library with probes derived from C5 yielded a number of cDNA clones which overlapped with the 5' end of clone C5 and extended further upstream (clones H9, H12, H17 and H3). In

parallel, two clones encoding the 3' end and poly-(A)$^+$ tail of the *Limk1* cDNA, clones M12 and M14, were isolated from a 16 day whole mouse embryo cDNA library using a probe derived from the 3'-end of clone C5. To ensure that the cDNA sequence obtained was derived from at least two independently isolated cDNA clones, additional clones of the region which lies between the H and M clones were obtained by RT-PCR of spinal cord mRNA (clones N1 and N2, see Figure 5). To reduce the risk of introducing mutations into the sequence during PCR amplification, N1 and N2 are the products of indepentent RT-PCR reactions using the Vent DNA Polymerase (New England Biolabs), which has a relatively low error rate of approximately one per 1000 nucleotides incorporated. The composite cDNA sequence of 3439bp and the deduced amino acid sequence for *Limk1* are shown in Figure 6. When we isolated the cDNA clones encoding the murine gene *Limk1*, no other sequence in the GenBank was identical to that of the newly isolated cDNA. However, as we proceeded with the characterisation of this gene, Mizuno *et al.* reported the cloning of a human cDNA sequence which they designated *LIMK* (Mizuno *et al.* 1994). The predicted amino acid sequences of *Limk1* and *LIMK* share 95% overall sequence identity, which strongly suggests that these two cDNAs are encoded by homologous genes. In agreement with the rules laid down by the mouse genome nomenclature committee, we have named the murine homolog, *Limk1*. The *Limk1* cDNA sequence has been submitted to the EMBL database (Accession number X86569).

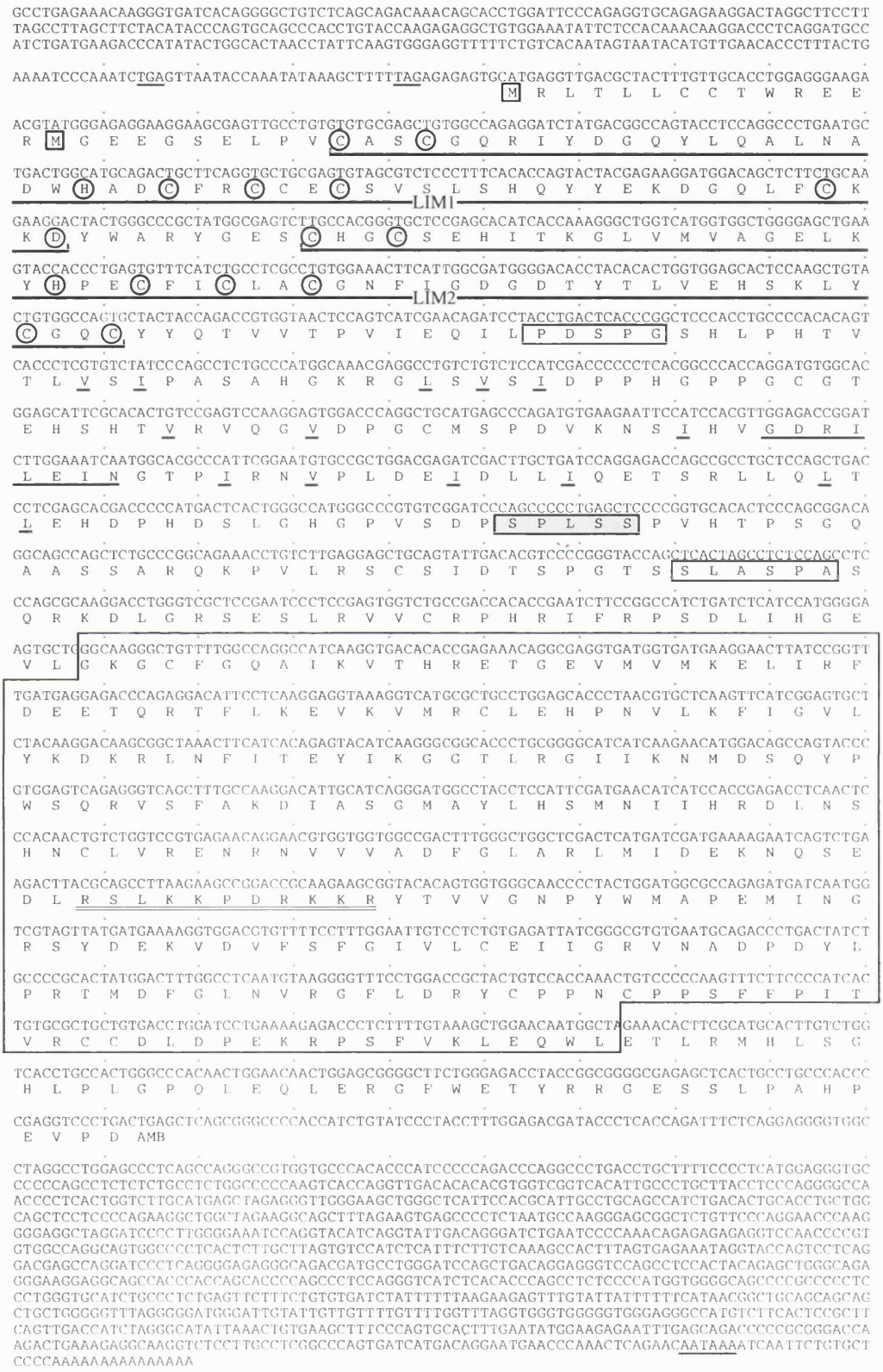

LIM1
LIM2

Figure 6: Composite cDNA and amino acid sequence of *Limk1*. LIM-domains (LIM 1, LIM 2) are delineated by straight brackets; circles mark conserved cysteine and histidine residues. Small squares highlight putative initiation codons; upstream stop codons are underlined. Putative MAP kinase phosphorylation sites are shown as open boxes; the putative casein kinase I phosphorylation site is shown as speckled box. Amino acid residues which align with conserved residues found in the DHR motif are underlined (between amino acid residues 165 and 257). The kinase domain is enclosed in a large irregular shaped box. The putative nuclear localisation signal is double underlined, the poly-adenylation signal single underlined.

Depending on which of two potential AUG initiation codons is used, the open reading frame encodes a protein of 646 or 632 amino acid residues, with a predicted molecular weight of approximately 72.7kD. No Kozak consensus sequence is present at either AUG (Kozak 1986; Kozak 1987), however two stop codons are found in-frame upstream of the first start codon. *Limk1* cDNA has a 322bp untranslated leader sequence and the open reading frame is followed by a 1172bp 3' untranslated region which contains a polyadenylation signal and poly(A)$^+$ tail (Proudfoot and Brownlee 1976).

3.2 Limk1 is a member of a novel class of LIM-domain kinases

When the predicted amino acid sequence of Limk1 was compared to known peptide sequence motifs found in other proteins, two important charcteristics of Limk1 were revealed. One is a pair of LIM domains arranged in tandem at the amino-terminus of Limk1, one at amino acid residues 25-75 (L1) and the other at residues 84-137 (L2) (Karlsson *et al.* 1990). Secondly, Limk1 contains a catalytic protein kinase domain located at the carboxy-terminal end, at amino acids 345-604 (Hanks *et al.* 1988; Hanks

and Quinn 1991). The intervening domain which connects LIM-domain and protein kinase domains, is rich in serine and proline amino acid residues (13% and 12% as opposed to 8% and 7%, respectively, within the entire protein). Some of these serine residues lie within sequence motifs which represent recognition sites for a number of serine/threonine kinases (Kemp and Pearson 1990; Pearson and Kemp 1991). Most prominent are putative phosphorylation sites for casein kinase I (SPLSS, aa 271-275) (Tuazon and Traugh 1991) and MAP kinase (LPDSPG, aa151-156; SLASPA aa307-313) (Gonzalez *et al.* 1991). Hydrophobicity plot analysis revealed no hydrophobic domains which could serve as either signal peptide sequence or transmembrane domain (Kyte and Doolittle 1982). Similarly, src-homology domains (SH2 or SH3) are also absent in Limk1 (Koch *et al.* 1991). However, a recent database search of protein sequences for the presence of the DHR motif (Ponting and Phillips 1995) revealed that LIMK may also contain a copy of the DHR motif within the intervening domain (amino acid residues 165 to 257, see Figure 6).

A comparison of the amino acid sequences of *Limk1* and *LIMK* revealed a high level of conservation between the murine and the human gene (overall 95% identity, Figure 7). The LIM and protein kinase domains of Limk1 show only conservative changes between the two species, while the intervening domain is slightly more divergent (92% identity). In particular the region surrounding the putative casein kinase I phosphorylation site appears to be less conserved. However, the putative casein kinase I and MAP kinase phophorylation sites are also present in LIMK. In addition, the region encompassing the putative DHR motif, which streches over most of the intervening domain, has also been highly conserved between *M. musculus* and *H. sapiens*. This conservation of the intervening domain stands in contrast to the divergence of the 5' and 3' untranslated cDNA sequences, with only 10% and 65% homology respectively. This suggests that the intervening

region is possibly more than just a linker between LIM and kinase domains, and may also perform other functions essential to the activity of Limk1.

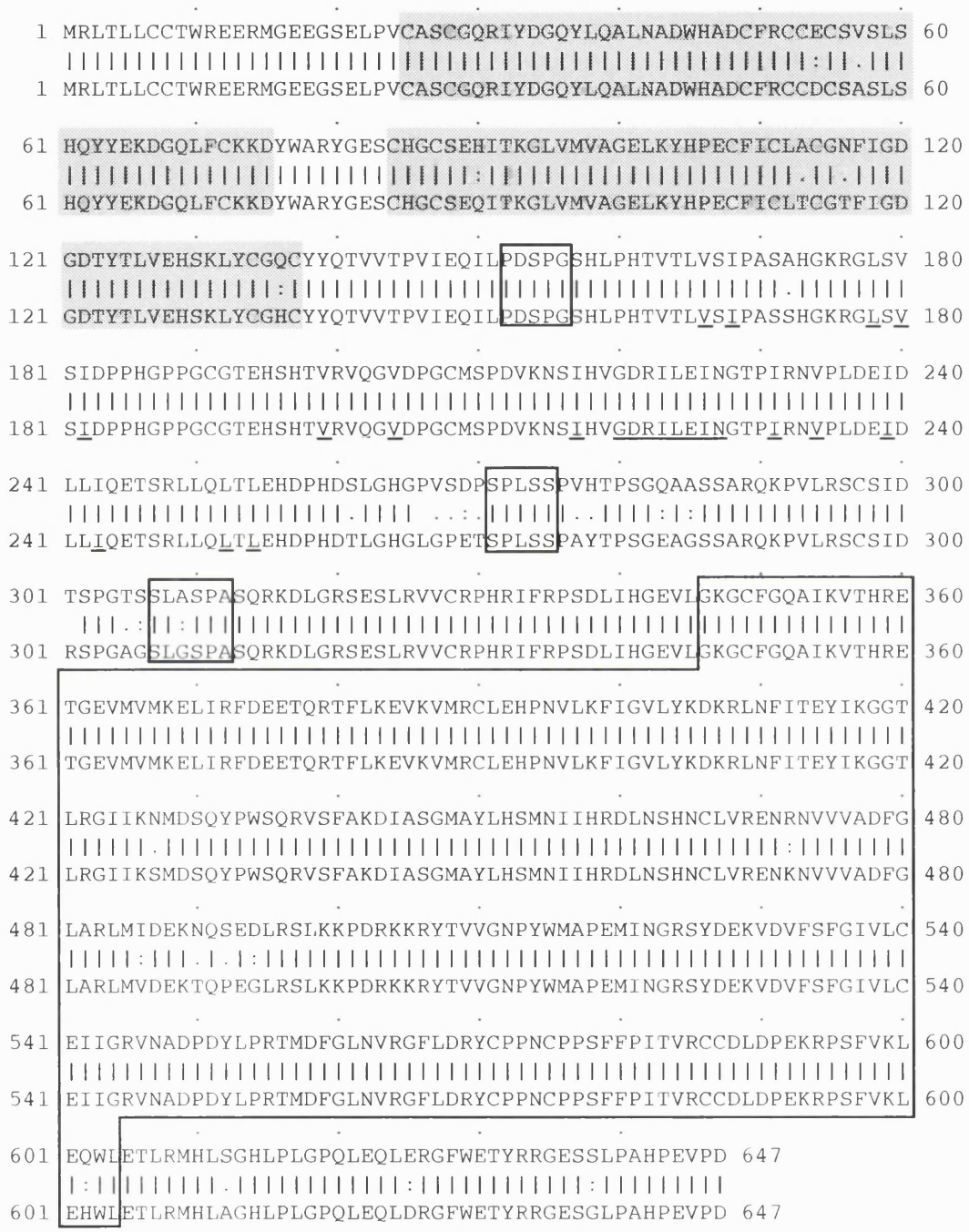
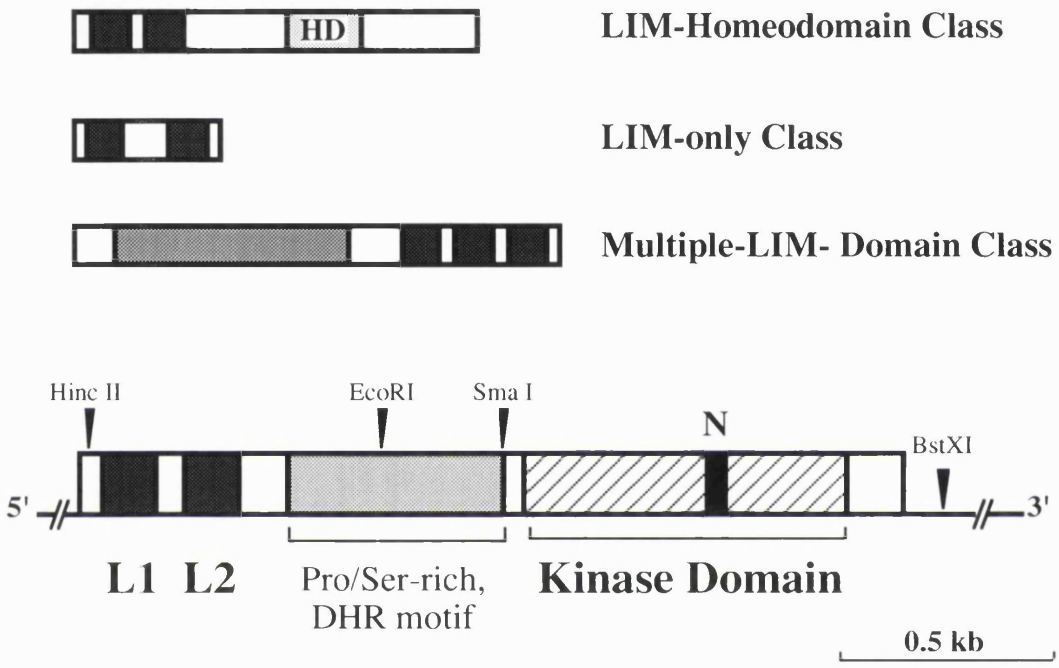


Figure 7: Comparison of murine Limk1 and human LIMK amino acid sequences. Overall sequence smilarity is 97%, overall sequence identity 95%. The Limk1 sequence is shown on the upper line, LIMK on the lower line. LIM domains 1 and 2 are highlighted by light grey boxes. Amino acids which are part of the putative DHR motif are underlined. Putative phosphorylation sites are enclosed in small boxes. The kinase domain is enclosed in a large, irregular box. Vertical bars represent amino acid identity, dots amino acid similarity. The alignment was generated according to Needleman and Wunsch (1970).

In Figure 8, the structural features of Limk1 are schematically outlined and compared to other classes of LIM-domain containing proteins. Clearly, Limk1 and LIMK define a new class of LIM-proteins distinct from either the LIM-homeodomain, LIM-domain-only or multiple-LIM-domain type proteins (Sanchez-Garcia and Rabbitts 1994).



Limk1 (LIM-Kinase Domain Class)

Figure 8: Schematic representation of the structural features of *Limk1* and comparison of *Limk1* with prototypes of other types of LIM-proteins. Dark boxes: individual LIM domains (L1: first double zinc-finger, L2: second double zinc-finger), hatched box: kinase domain, N: putative nuclear localisation signal, HD: homeodomain, dark grey box: other protein domains, e.g. vinculin/fak binding site, light grey box: proline/serine rich region. Hydrophobic signal peptide or transmembrane sequences were not detected, suggesting that Limk1 is an intracellular protein. The serine and proline rich intervening domain contains putative casein kinase I and MAP kinase phosphorylation sites as well as a putative DHR motif. Furthermore, a short and highly basic insert (RSLKKPDRKKR) is present within the kinase domain. This sequence may act as a putative nuclear localisation signal (Chelsky *et al.* 1989). Unlike most kinase domain inserts which are located between subdomains V and VIa, this short insert lies between subdomains VIa and VIb.

In order to determine the place of Limk1 within the rapidly growing families of LIM-proteins and protein kinases, the amino acid sequences of these domains were used to construct sequence alignments and phylogenetic trees. As in the majority of LIM-domain proteins, the two double zinc finger/LIM motifs found in Limk1 were arranged in tandem. In Limk1 these were separated by a short eight amino acid spacer. Aligning the LIM domains of Limk1 with the LIM-domain consensus sequences (Figures 2 and 9) revealed a complete conservation of the cysteine and histidine residues, believed to be involved in metal-binding (Michelsen *et al.* 1994; Perez-Alvarado *et al.* 1994). A number of other hydrophobic or basic amino acid residues which are conserved among a majority of LIM-proteins from different species were also present in Limk1 (Wang *et al.* 1992).

```
        1      * *       *    *              *                51
Limk1   CASCGQRIYD GQYLQALNAD WHADCFRCCE CSVSLSHQYY EKDGQLFCKKD
LIM1    C..C...I.D ......L... WH..C..C.. C...L.............C..D

        1           *         *                   *           54
Limk2   CHGCSEHITK GLVMVAGELK YHPECFICLA CGNFIGDGDT YTLVEHSKLYCGQC
LIM2    C..C...... .L........ YH..C..C.. C.......D. ..........C..C
```

Figure 9: Alignment of Limk1 LIM-domains with the LIM-domain consensus sequence (LIM1: first, LIM2: second LIM domain). Limk1 contains all conserved amino acid residues believed to be involved in zinc ion binding (bold). Other hydrophobic and basic residues are also conserved (asterisk).

The phylogenetic analysis of LIM-domains showed that Limk1 and LIMK cluster within the LIM-homeodomain class of proteins (examples shown: apterous, Isl1, lmx-1, LH2, lin-11, mec-3, Xlim-1 and -3; Figure 10, square box). The majority of LIM-only proteins form a seperate group (examples shown: CRP, CRP2, CRIP, MLP; Figure 10, rounded boxes), while the LIM-domains of Zyxin and Paxillin belong to neither of these two subfamilies (Sanchez-Garcia and Rabbitts 1994).

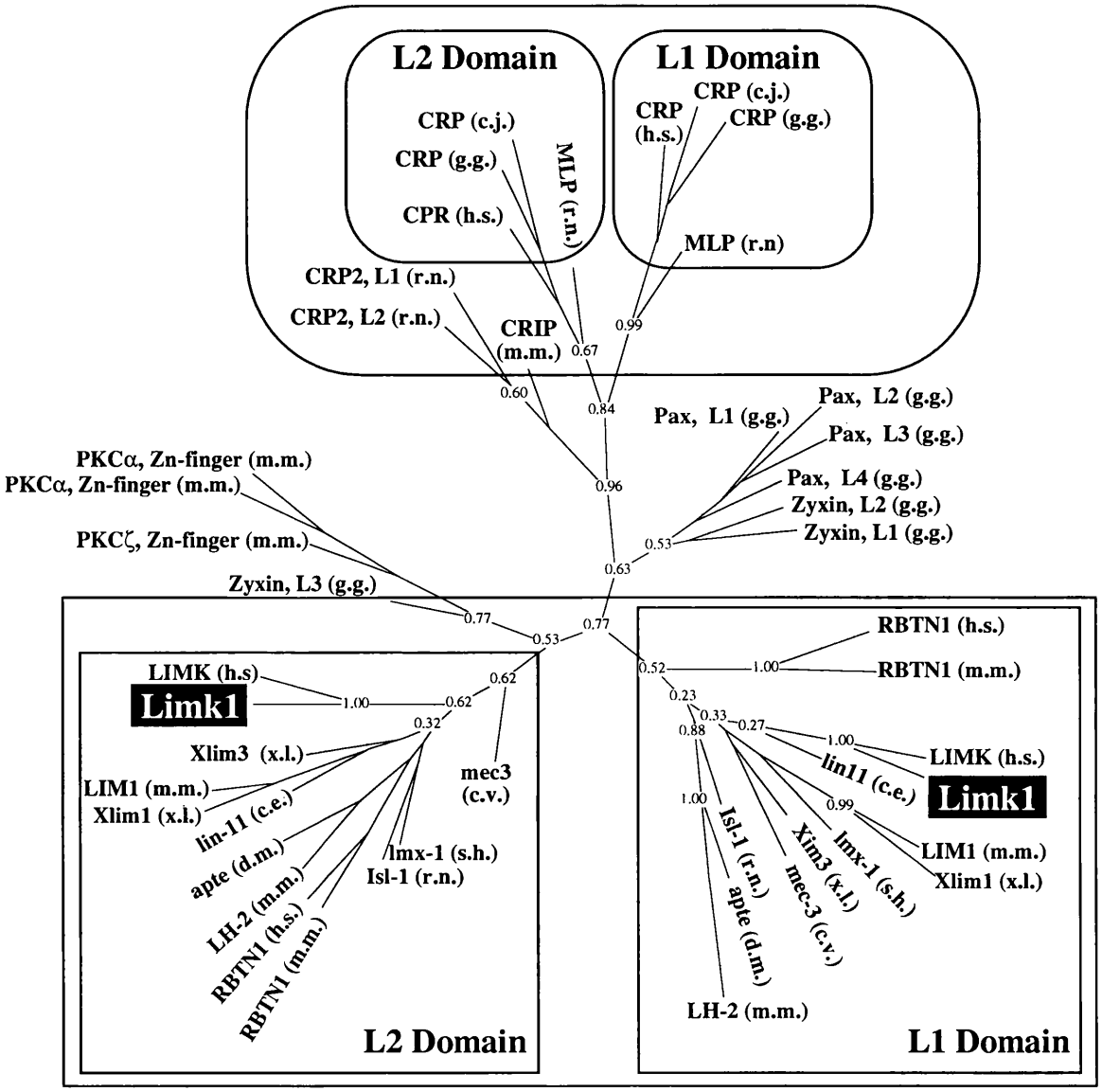


Figure 10: Comparison of Limk1 LIM-domains to those of other known LIM-proteins. The phylogenetic dendrogram of separate LIM-domains (L1, L2, etc.) of most known LIM-proteins based on amino acid sequence. The large rounded box encompasses the cysteine-rich protein subclass of LIM-proteins, while the square box contains members of the LIM-homeodomain subfamily, the Rhombotins and Limk1. Zyxin and Paxillin appear to belong to neither group. The position of Limk1 is highlighted in bold. Relative bootstrap values are shown (maximum 1.00, minimum 0). Because this tree is not rooted, the zinc-finger domains of PKC α and ζ have been included as examples of zinc fingers which are not involved DNA binding. Dendrogram was generated using Clustal V and Phylip programs. Abbreviations: c.e., *Caenorhabditis elegans*; c.v. *Caenorhabditis vulgaris*; c.j. *coturnix japonica*; d.m., *Drosophila melanogaster*; g.g., *Gajus gajus*; h.s., *Homo sapiens*; m.m., *Mus musculus*; r.n., *Rattus norvegicus*; s.h., syrian hamster; x.l., *Xenopus laevis*..

Similarly, Figure 11 shows a phylogenetic comparison of the Limk1 kinase domain to a selection of protein kinases, which includes representatives from the tyrosine kinase family (box), the serine/threonine kinase family (cAPK, PKC, cdc2, raf, mos, ACTR) and putative dual specificity kinases (Lindberg *et al.* 1992). Limk1 appeared to be closest related to raf-1, a serine/threonine kinase and DPYK-1 and -2, both putative dual specificity kinases. However, because the bootstrap probability was relatively low at this branch (0.27), the exact relationship between Limk1, raf-1 and DPYK is unclear. Overall, Limk1 and LIMK were positioned close to the interface between serine/threonine and tyrosine kinases, indicating that Limk1 may not encode either a typical serine/threonine or tyrosine kinase.

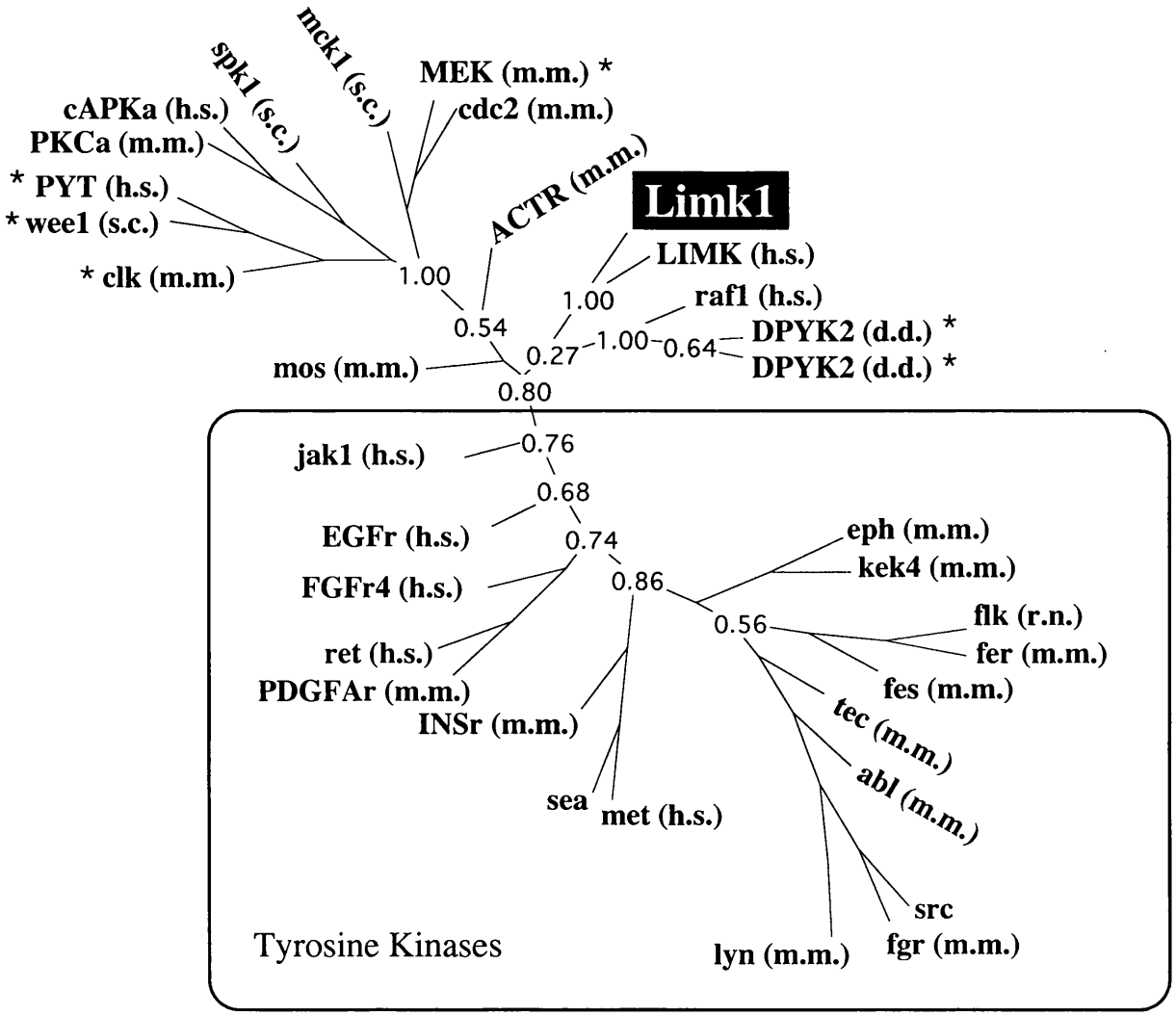


Figure 11: Comparison of Limk1 kinase domain to the kinase domains of other protein kinases. The phylogenetic dendrogram of selected kinases represents protein tyrosine kinases (box), serine/threonine kinases, and putative dual-specificity kinases (asterisk). The position of Limk1 is highlighted. Relative bootstrap values are shown (maximum 1.00, minimum 0). This tree is not rooted. Dendrogram was generated using Clustal V and Phylip programs. Abbreviations: d.d. *Dictyostelium discoidum*; h.s., *Homo sapiens*; m.m., *Mus musculus*; r.n., *Rattus norvegicus*; s.c., *Saccheromyces cerevisiae*.

Indeed, a sequence alignment of catalytic kinase domains between Limk1 and other known protein kinases, highlighted the unusual nature of the Limk1 kinase domain. The alignment shown in Figure 12 was based on the previously described subdomain structure of catalytic domains found in protein kinases (see section 1.2.3 Hanks *et al.* 1988; Hanks and Quinn 1991).

Alongside of Limk1, the consensus sequences of protein serine/ threonine kinases (PSK) and protein tyrosine kinases (PTK) are shown in Figure 12. While Limk1 did contain all of the highly conserved amino acid residues defined within the kinase domain, some differences were found, particularly in subdomains VIb and VIII. These domains are part of the catalytic core and have been previously used to predict the hydroxy-amino acid specificity of kinases (Hanks *et al.* 1988). The Limk1 sequence in subdomain VIb (HRDLNSHN) and VIII (GNYWMAPE), aligned only partially with either the PSK or PTK consensus motifs. The region surrounding the highly conserved lysine in subdomain II also failed to align exactly to either the serine/threonine or tyrosine kinase consensus. In addition, neither of these regions in Limk1 showed any significant homology to any of the putative dual specificity kinases (Lindberg *et al.* 1992), which places Limk1 together with LIMK in a distinct class of protein kinases.

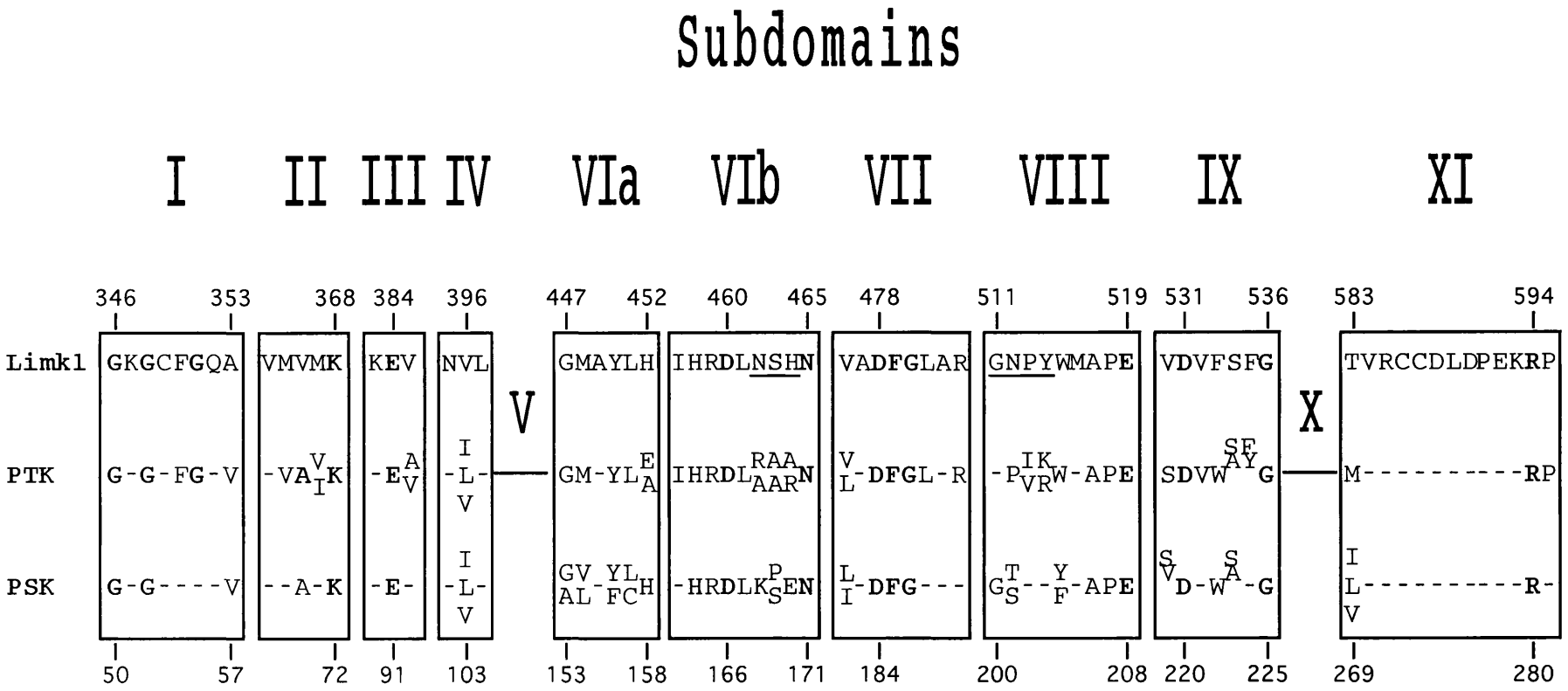


Figure 12: Alignment of conserved kinase subdomains: the amino acid sequence of Limk1 is aligned to the consensus sequences of protein tyrosine kinases (PTK) and protein serine/threonine kinases (PSK). Amino acid residues are represented in single letter code. Except for few highly conserved amino acid residues, subdomains III, IV, V, VIb, X and XI only exhibit common sequence motifs among subfamilies within the tyrosine or serine/threonine kinase families. Amino acid enumeration at the top refers to the Limk1 peptide sequence, enumeration at the bottom refers to cyclic-AMP dependent kinase α (see Hanks *et al.*, 1988). Dash represents variable residues. Limk1 contains all conserved amino acids which have been shown to be necessary for kinase activity. However, Limk1 differs from both the PTK and PSK consensus (underlined residues) in regions diagnostic of either PSK or PTK's (see section 1.2.3 for details).

3.3 Limk1 fusion protein exhibits serine/threonine and tyrosine protein kinase activity in vitro

In view of the unusual kinase domain sequences found in subdomain VIb, it was necessary to test the kinase activity of Limk1 *in vitro*. Overexpression of the protein kinase domain as a fusion protein is often sufficient to constitutively activate kinase activity, which then leads to auto-phosphorylation (Fantl *et al.* 1993). Therefore the C-terminal half of *Limk1*, which contains the kinase domain, was expressed in either bacteria or baculo virus as a recombinant glutathione-transferase (GST)-Limk1KD fusion protein (Figure 13). These constructs also contained parts of the serine/proline rich region linking LIM and kinase domains.

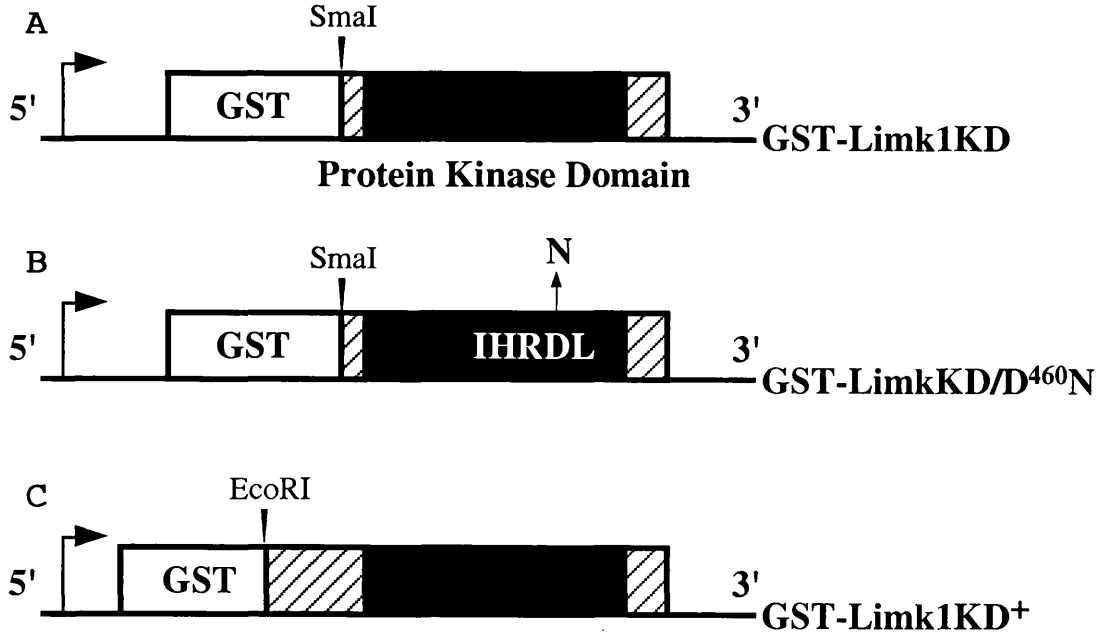


Figure 13: To test the *in vitro* kinase activity of Limk1, the kinase domain of Limk1 was expressed as a recombinant glutathione-transferase fusion protein, both in bacteria (construct A and B: Limk cDNA fragment from nucleotide 1230 to 2368 cloned in frame into expression vector pGEX) and in Sf9 insect cells (construct C: Limk cDNA fragment from nucleotide 964 to 2368 cloned in frame into baculovirus expression vector pAc3GX). The predicted size of th fusion proteins is approximately 68kD (A and B) and 80kD (C). To generate a kinase negative mutant, D460 in the IHRDL motif of subdomain VIb was mutatedto asparagine (construct B, GST-Limk1KD/D460N).

In the absence of an antibody specific for the carboxy-terminal end of Limk1, the expected molecular weight of 80kD for the fusion protein GST-Limk1KD+ was confirmed by anti-GST western blot (Figure 14A). The in vitro kinase assay of the purified GST-Limk1KD$^+$ fusion protein, bound to glutathione-sepharose beads, showed auto phosphorylation in the presence of either 5mM Mg^{2+} or 5mM Mn^{2+} ions (Figure 14B, Lanes 1 and 2). In the presence of both 5mM Mn^{2+} and 5mM Mg^{2+}, levels of auto phosphorylation increased in an additive manner (Figure 14B, Lane 3). No phosphorylated proteins were present in polyhedrin-negative baculovirus control extracts following affinity purification with glutathione-beads (Figure 14B).

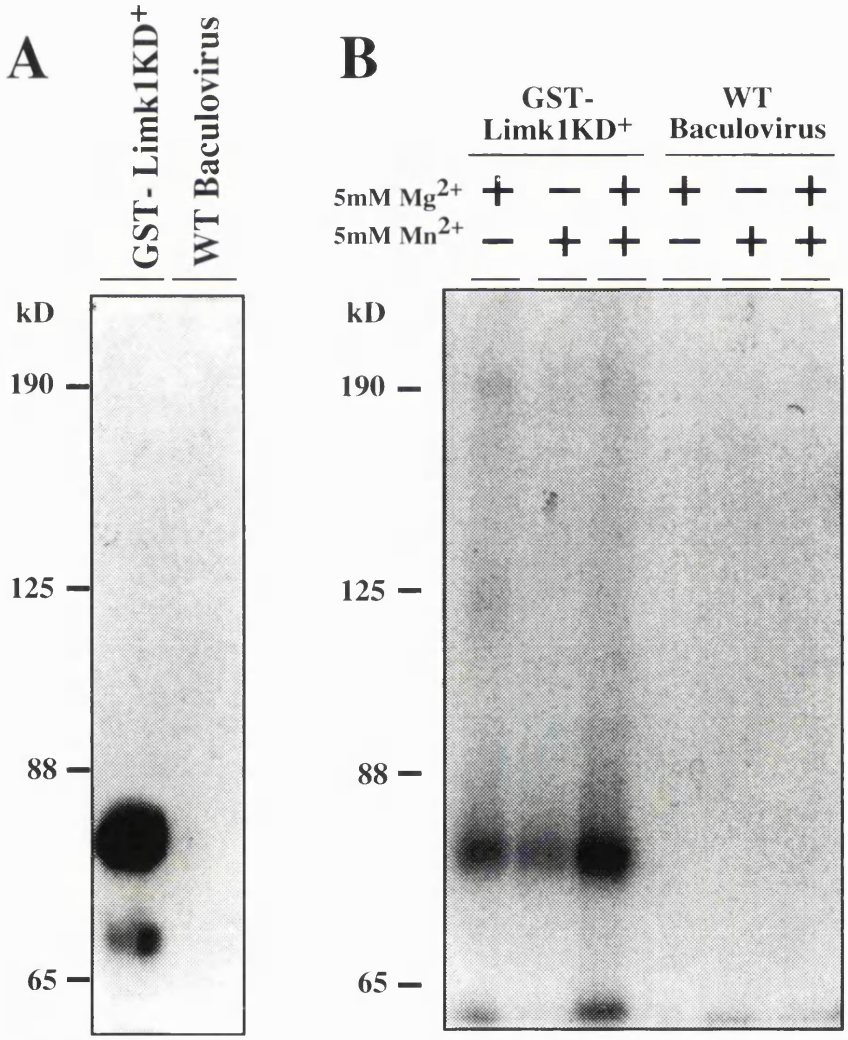


Figure 14: The kinase domain of Limk1 exhibits protein kinase activity. (**A**) Anti-GST western blot confirmed the predicted size of ca. 80kD for the GST-Limk1KD fusion protein synthesised in baculovirus. (**B**) The GST-LimkKD fusion protein was phosphorylated in an in vitro kinase assay, in the presence of Mn^{2+} or Mg^{2+} ions.

To exclude the possibility that co-purifying proteins may be responsible for the phosphorylation of GST-Limk1KD fusion protein, an Asp460 to Asn point substitution was introduced into the Limk1 kinase domain (GST-LimkKD/D460N). This mutation lies within the HRDL motif of the kinase domain and has been shown to completely abolish kinase activity in both cytoplasmic and receptor tyrosine kinases (Moran *et al.* 1988; Reith *et al.* 1991; Reith *et al.* 1993). The *in vitro* kinase assay performed with the GST-LimkKD/D460N mutant protein showed no auto phosphorylation (Figure 15A), while similar amounts of fusion protein were present in both reactions (Figure 15B). The approximately 90kD band visible in both lanes is an artefact also present in extracts of bacteria expressing only the pGEX3 vector.

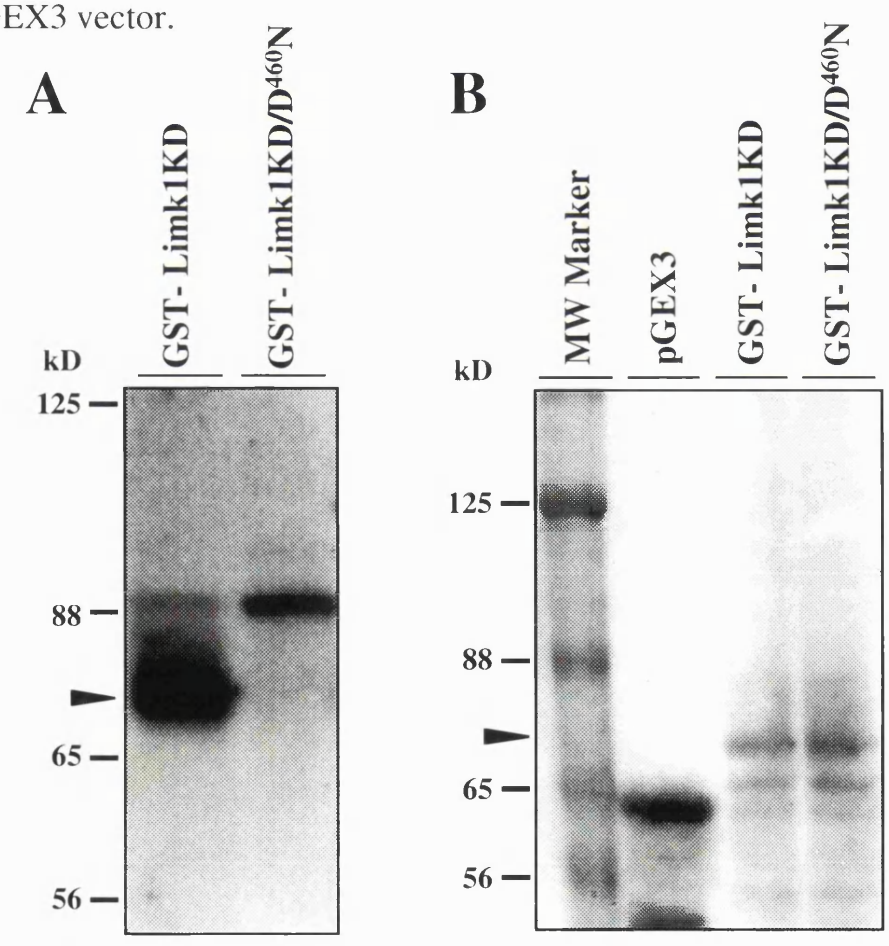


Figure 15: Mutation of Asp460 in the kinase domain of Limk1 abolishes kinase activity. (**A**) The kinase activity of Limk1 was abolished when the D460→N mutation was introduced into the kinase domain of the GST-Limk1KD protein. (**B**) Coomassie stain of SDS-gel loaded in parallel with glutathione affinity purified protein extracts reveals similar amounts of GST-Limk1KD and GST-Limk1KD/D460N fusion proteins.

In order to gain some understanding of the type of kinase activity exhibited by Limk1, wild type GST-Limk1KD$^+$ protein was affinity purified from Sf9 cells and probed with an anti-phosphotyrosine (anti P-Tyr) antibody (Figure 16A). The fusion protein was found to be phosphorylated on tyrosine residues.

To test the in vitro hydroxy-amino acid specificity of the Limk1 kinase domain, phosphoamino acid analysis was performed after an in vitro kinase reaction using the purified fusion protein (Figure 16B). The highest level of phosphorylation was detected on serine residues, followed by phospho-tyrosine and trace amounts of phospho-threonine. The ability of Limk1 to use all three hydroxy amino acids as acceptor sites in vitro again highlights the unusual nature of the Limk1 kinase domain.

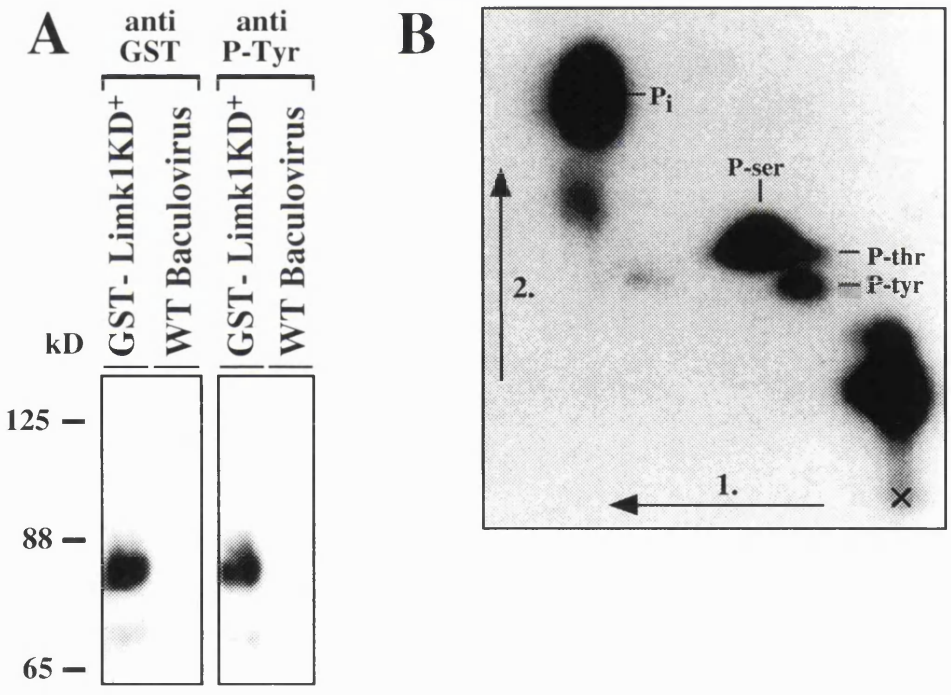


Figure 16: The Limk1 kinase domain exhibits serine/threonine and tyrosine kinase activity in vitro. (**A**) Anti-phosphotyrosine western blot of GST-Limk1KD$^+$ fusion protein affinity purified from Sf9 extracts. Anti-glutathione transferase western blot identifies fusion protein. (**B**) Two-dimensional phosphoamino acid analysis of GST-Limk1KD fusion protein reveals a high level of in vitro phosphorylation on serine residues (p-ser) followed by phosphotyrosine (p-tyr) and trace amounts of phosphothreonine (p-thr); pi free phosphate, x: origin, arrows: first and second dimensions of TLC.

3.4 *Limk1* maps to mouse chromosome 5

To determine whether, *Limk1* was a single copy gene, high stringency Southern blot analysis was performed on genomic DNA from *Mus musculus* C57BL/6 and DBA mouse strains, which had been digested with the restriction endonuclease *Sal*I and size fractionated on an agarose gel. When probed with two different, non-overlapping fragments of *Limk1* cDNA, a single large DNA fragment was detected in genomic DNA isolated from *M. musculus* strain C57Bl/6J (Figure 17). Although this suggested that *Limk1* is a single copy gene, pulse-field gel electrophoresis would be required to confirm the presence of a unique band in these preparations.

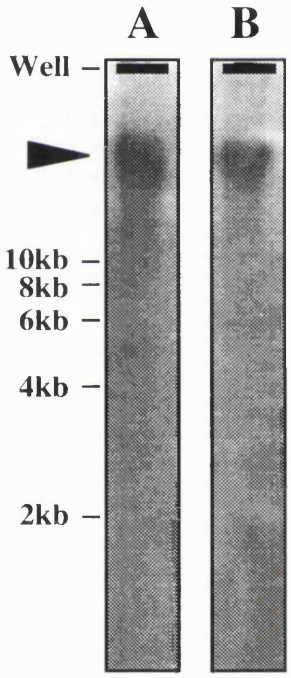


Figure 17: *Limk1* is a single copy gene in Mus musculus C57Bl/6J. High stringency Southern blot analysis of *Sal*I digested genomic DNA reveals a single band of equal size when probed with cDNA probes derived from either the 5' end (**A**: *Hinc*II-*Eco*RI fragment) or from the 3' half (**B**: *Sma*I-*Bst*XI fragment) of *Limk1* cDNA.

The chromosomal location of *Limk1* was determined by interspecific backcross analysis using progeny derived from a ([*Mus musculus* (C57BL/6J) x *Mus spretus*]F1 x *Mus spretus*) backcross panel. This mapping panel has been typed for over 1230 loci (Rowe *et al.* 1994). C57BL/6J and *Mus spretus* DNA were digested with several restriction endonucleases and the products analysed by Southern blot hybridisation using a 2kb *Eco*RI fragment of clone C5. When genomic DNA was digested using *Bgl*II, this cDNA probe detected a restriction fragment length polymorphism (RFLP) between C57BL/6J (DNA fragments sized 2.2kb, 2.9kb, 3.3kb, 4.3kb) and *Mus spretus* DNA (DNA fragments sized: 2.9kb, 3.3kb and 6.5kb). The 4.3kb C57BL/6 and 6.5 kb *Mus spretus* fragmens were then used to follow the segregation of the *Limk1* locus in 94 backcross mice (Figure 18). The segregation pattern obtained was then compared to those of previously mapped loci, and gene order determined by minimizing the number of recombination events required to explain allele distribution patterns. Thirty single and one double recombinant chromosomes were identified (Figure 19A). The mapping data indicates that *Limk1* is located on the distal end of chromosome 5 and is linked to *Nos*1, *Zp*3 and *Ipf*1. A partial map of chromosome 5 is depicted in Figure 19B. The gene order and recombination frequencies, expressed as genetic distances in centi-Morgans (cM) ± standard error, are: centromere-//-D5mit9-(9.57±3.03)-*Nos*1-(9.57±3.03)-D5Hun11-(3.19±1.81)-*Limk*1-(3.19±1.81)-*Zp*3-(2.13±1.49)-Pmv12-(6.38±2.52)-*Actb*.
The chromosomal mapping data has been deposited in the Mouse Genome Databank, Jackson Laboratory (Accession No. MGD-CREX-250). Loci beginning with the code D5 represent markers identified solely on the basis of chromosomal position. Unless indicated otherwise, these are not associated with known transcriptional units. The genes encoding the following proteins have previously been mapped to the distal end of chromosome 5: T-box gene 3 (Tbx3, Bollag *et al.* 1994), mixed-function

oxidase component 1 (Nos1, Simmons *et al.* 1985), zona pellucida protein 3 (Zp3, Lunsford *et al.* 1990), insulin promoter factor (Ipf1, Leonard *et al.* 1993), and cytoplasmic β actin (Actb, Czosnek *et al.* 1983). *Pmv-12* and *Iapls3-9* indicate the map positions of endogenous provirus sequences (Frankel *et al.* 1989; Lueders *et al.* 1993). Both *Ipf1* and *Zp3* (van Duin *et al.* 1993) have been mapped to human chromosome 7q21.3-q22.1 and 7q21 respectively, indicating that these two chromosomal regions are homologous in mouse and man (Nadeau and Taylor 1984). This suggests that the human homologue of *Limk*1 may lie near chromosome 7q22.

[(*Mus musculus* C57Bl/6 x *Mus spretus*) x *Mus spretus*] Panel DNA

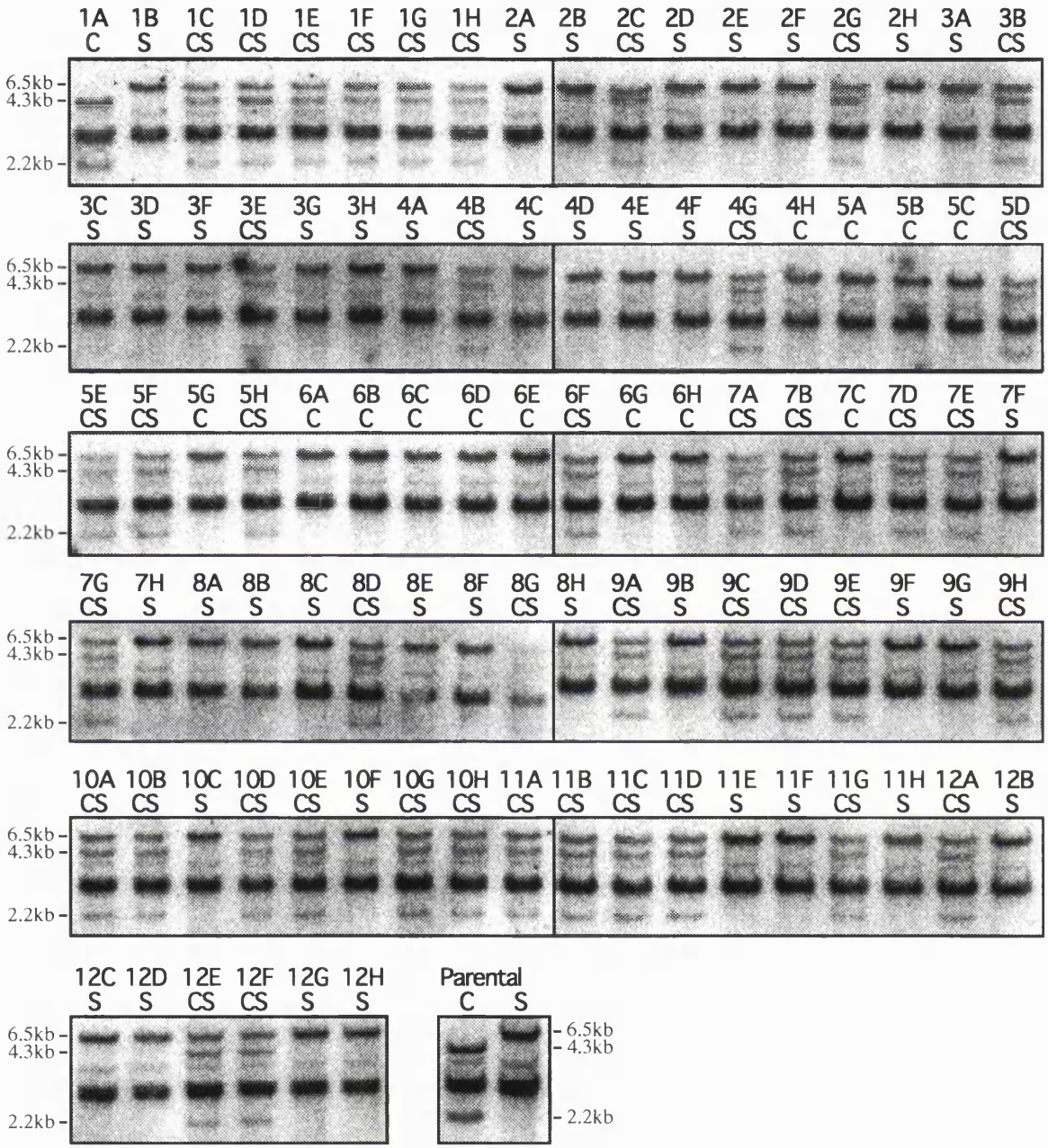


Figure 18: Southern blot analysis of *Bgl*II digested genomic DNA reveals the segregation pattern of a 4.3kb and 2.2kb C57BL/6, and a 6.5 kb *Mus spretus* specific DNA fragment, when hybridised with a *Limk1* specific cDNA probe (cDNA clone C5). C, C57BL/6 homozygote; S, Mus spretus homozygote, CS, C57BL/6 x Mus spretus heterozygote genotype. Backcross offspring were enumerated 1A-H, 2A-H, etc.

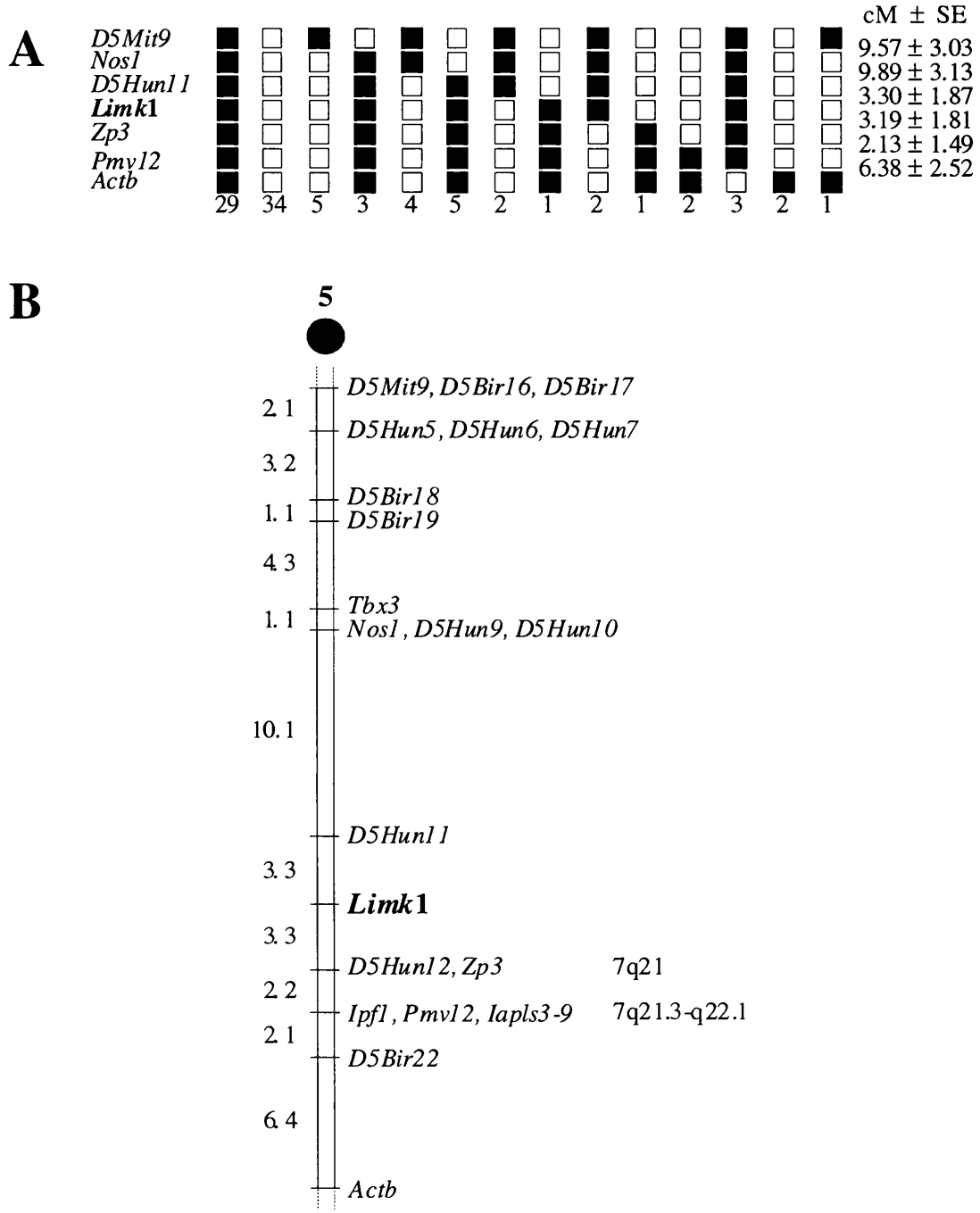


Figure 19: Chromosomal location of *Limk1*. (**A**) The segregation patterns of *Limk1* and flanking genes in 94 backcross progeny are shown. Each column represents the chromosome inherited from the [C57BL/6J x *Mus spretus*]F1 parent. Closed boxes represent the C57BL/6J allele, open boxes the *Mus spretus* allele. The total number of offspring to inherit each type of chromosome is listed below each column. (**B**) The partial linkage map of mouse chromosome 5 depicts the order of loci and recombination distances between loci, in centiMorgans to the left. The equivalent positions of loci which have also been mapped on the human chromosome map are listed on the right.

3.5 *Limk1* mRNA is predominantly expressed in the nervous system

Expression of *Limk1* was analysed by both northern blot analysis and *in situ* hybridisation. Using a 0.8kb *Hinc*II/*Sma*I cDNA probe, which spans the LIM domains, a 3.5kb mRNA was detected by northern blot. In the adult mouse, *Limk1* appeared to be preferentially expressed in the nervous system (Figure 20; spinal cord, cortex, cerebellum). High levels of *Limk1* mRNA were also present in placenta, while weaker levels of expression were observed in thymus, ovaries, uterus, testis and lung (Figure 20). Longer exposure revealed a second, very low level transcript of approximately 6.5kb in testis. However, unlike *LIMK*, expression of *Limk1* was not detected here in liver, kidney or heart (Mizuno *et al.* 1994).

Limk1 was also expressed as a 3.5kb mRNA in midgestation mouse embryos (Figure 21, day 9.5, day 10.5 and day 12.5 p.c. whole mouse embryos). Total RNA isolated from microdissected neuroepithelium of day 9.5 p.c. embryos revealed a high level of *Limk1* expression when compared to levels detected in total RNA from whole embryos (Figure 21), suggesting that *Limk1* may already be preferentially expressed in the developing nervous system of the embryo. *Limk1* expresion was also detected in F1 melanoma cells derived, from murine keratinocytes, and in the embryonic stem cell line D3. As expected, *Limk1* was expressed in the embryonic liver cell line tsA58-L25. However, *Limk1* appeared was not detected in either adult (Figure 20) or embryonic day 13 p.c. liver (data not shown).

One major cell type found in the nervous system are glial cells, which include type-1 astrocytes, oligodendrocytes (in the central nervous system), Schwann cells (in the peripheral nervous system) and the precursors of oligodendrocytes, the O-2A progenitor cells. Purified cultures of primary rat

glial cells were also tested for the expression of *Limk1* (Figure 22). Total RNA was from isolated from 95% pure cultures of Schwann cells derived from adult sciatic nerve, 95% pure cultures of O-2A progenitor cells derived from postnatal day 7 optic nerve, pure postnatal day 1 cortical type-1 astrocyte cultures and pure postnatal day 7 oligodendrocyte cultures. *Limk1* was found to be expressed in all of these glial cell-types (Figure 22). When taking into account the relative levels of RNA loaded on to the denaturing gel (Figure 22B), expression levels appeared to be similar among Schwann cells, O-2A progenitor cells and type-1 astrocytes, with slightly increased levels of *Limk1* found in oligodendrocytes. Expression was also found in cultures of postnatal day 7 meningeal cells isolated from the optic nerve and in proliferating PC12 cells cultivated in serum (Figure 22).

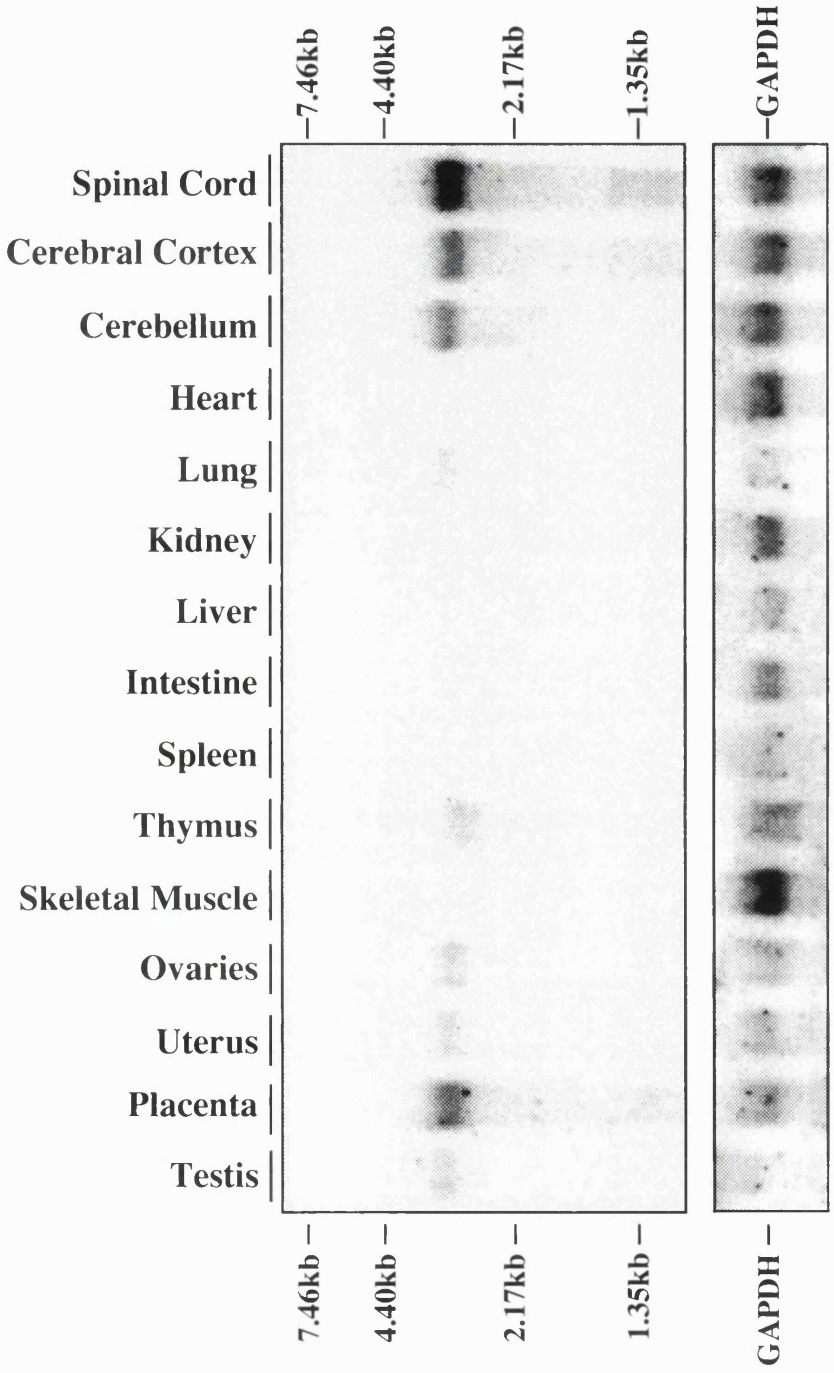


Figure 20: Northern blot analysis of *Limk1* expression in adult mouse tissues. 15µg of total RNA were loaded per sample. The blot was hybridised with either a 0.8kb *Hinc*II/*Sma*I fragment from the amino-terminal of *Limk1* (left panel) or with a full-length cDNA probe of murine GAPDH (right panel).

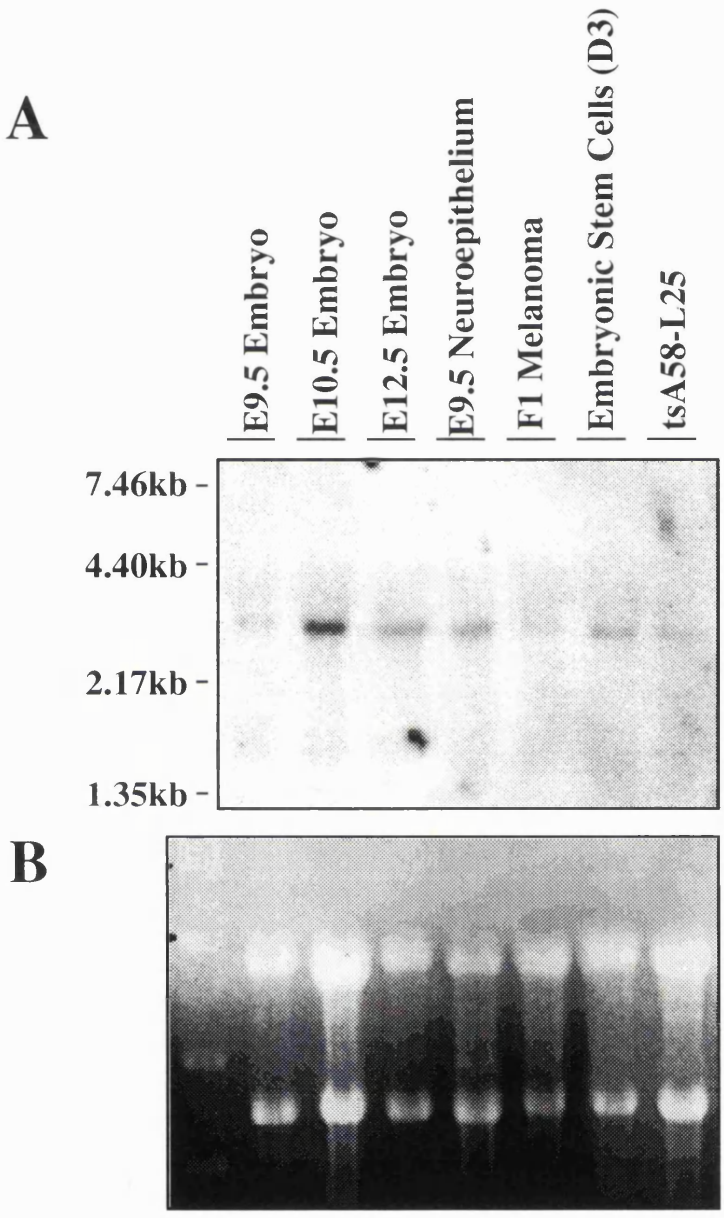


Figure 21: Northern blot analysis of *Limk1* expression in mouse embryos. 15µg of total RNA were loaded per sample. Blots were hybridised with a 0.8kb *Hinc*II/*Sma*I fragment from the amino-terminal of *Limk1* cDNA. (**A**) Northern blot of mouse embryo RNA, embryonic neuroepithelium, F1 melanoma , D3 embryonic stem cell and embryonic liver cell line (tsA58-L25) cultures. (**B**) The ethidium bromide stained agarose gel of blot A shows equivalent amounts of RNA loaded in all samples.

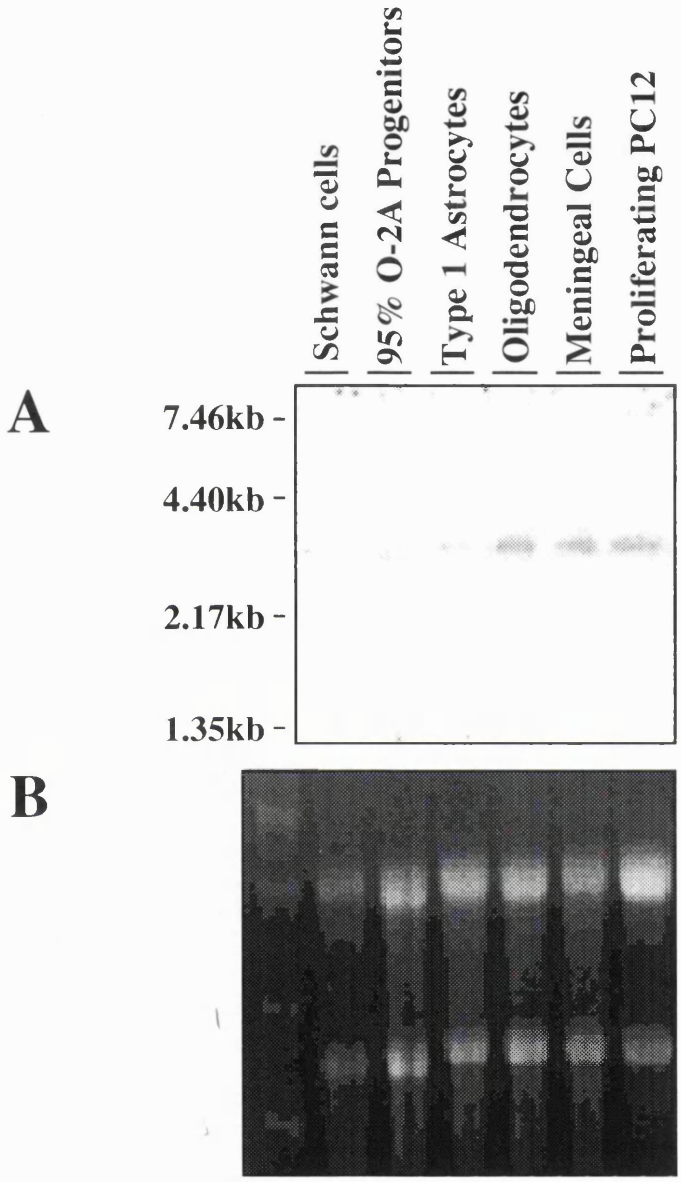


Figure 22: Northern blot analysis of *Limk1* expression in cultures of rat glial cells. 15µg of total RNA were loaded per sample. Blots were hybridised with a 0.8kb *Hinc*II/*Sma*I fragment from the amino-terminal of *Limk1* cDNA. (**A**) Northern blot of cultures of different glial cell types isolated from rat tissues. Specifically: ca. 95% pure cultures of Schwann cells derived from adult sciatic nerve, 95% pure cultures of O-2A progenitor cells derived from postnatal day 7 optic nerve, pure postnatal day 1 cortical type-1 astrocyte cultures and pure postnatal day 7 oligodendrocyte cultures. (**B**) The ethidium bromide stained agarose gel of blot A shows equivalent amounts of RNA in all samples except.

The spatial and temporal distribution of *Limk1* mRNA during development was studied using *in situ* hybridisation. Throughout the second half of embryonic development and in the adult, expression was most prominent along the entire length of the spinal cord. At day 10.5 post coitum (p.c.) *Limk1* message was detected in the neural tube along the entire length of the embryo (whole mount *in situ* hybridisation; Figure 23A). Already at this stage expression appeared to be localised to the ventral half of the neural tube (Figure 23B). Expression of *Limk1* in the cephalic vesicle of the forebrain appeared to be present in both alar and basal plate (Figure 23C).

The distribution of *Limk1* mRNA along the dorso-ventral axis of the neural tube is more obvious in cross sections of embryos from day 11.5 p.c. (Figure 24). At this stage increased levels of *Limk1* mRNA were detected in the ventrolateral regions and in the area near the basement membrane of the spinal cord. *Limk1* was also expressed in the developing dorsal root ganglia which arise from the neural crest derived spinal primordia (Figure 24C). By day 14.5 p.c. increased levels of expression were still present in the ventral horns of the spinal cord. However *Limk1* was now also expressed in most of the mantle layer of the spinal cord, with exception of the ventricular zone and the dorsal horns (Figure 25A). This distribution in the spinal cord persists at least until day 18.5 p.c. (data not shown). The areas which show a higher level of *Limk1* expression appear to correlate with regions in which the median motor column (MMC) and lateral motor colomns (LMC) form in the chick spinal cord (Hollyday and Hamburger 1977). However, in the young adult mouse, *Limk1* is expressed in large cells or groups of cells which are evenly distributed throughout the grey matter of the spinal cord, and does not show elevated levels of expression in the ventral horns (Figure 25C).

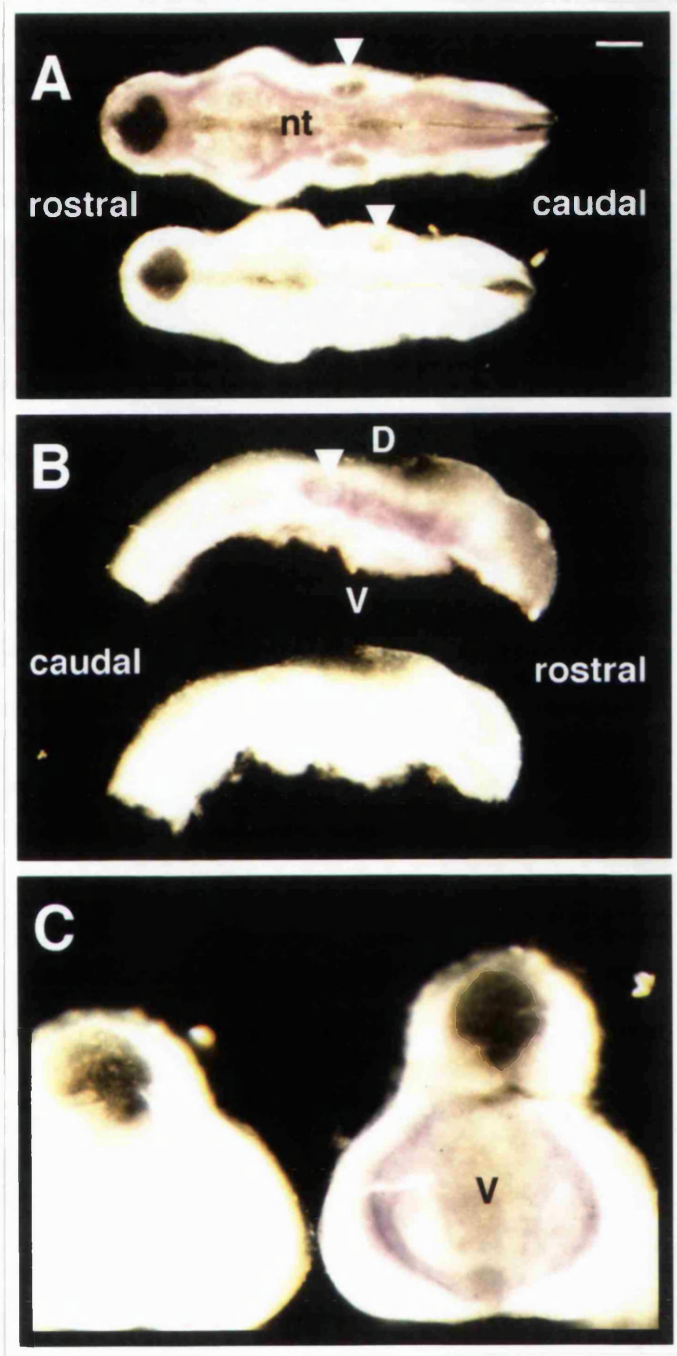


Figure 23: Distribution of *Limk1* mRNA in the neural tube of E10.5 mouse embryos. Whole mount in situ hybridisation with a Limk1 cRNA antisense probe (upper specimen in panels A and B; right specimen in panel C) and sense probe (lower specimen in panels A and B, left specimen in panel C) labelled with DIG-UTP and visualised using anti-DIG alkaline phosphatase antibody. (**A**) Dorsal view of E10.5 embryos after removing fore- and midbrain vesciles, as well as ventral structures (heart, foregut, hindgut) and tail. A: anterior, P: posterior, arrow: otic vesicle, nt: neural tube (**B**) Lateral view of specimen shown in panel A. Labels as in A. D: dorsal, V: ventral. (**C**) Frontal view of forebrain vesicle. Labels as in A. V: vesicle lumen. Scale in (A) bar represents: 1.5 mm (A,B), 1mm (C).

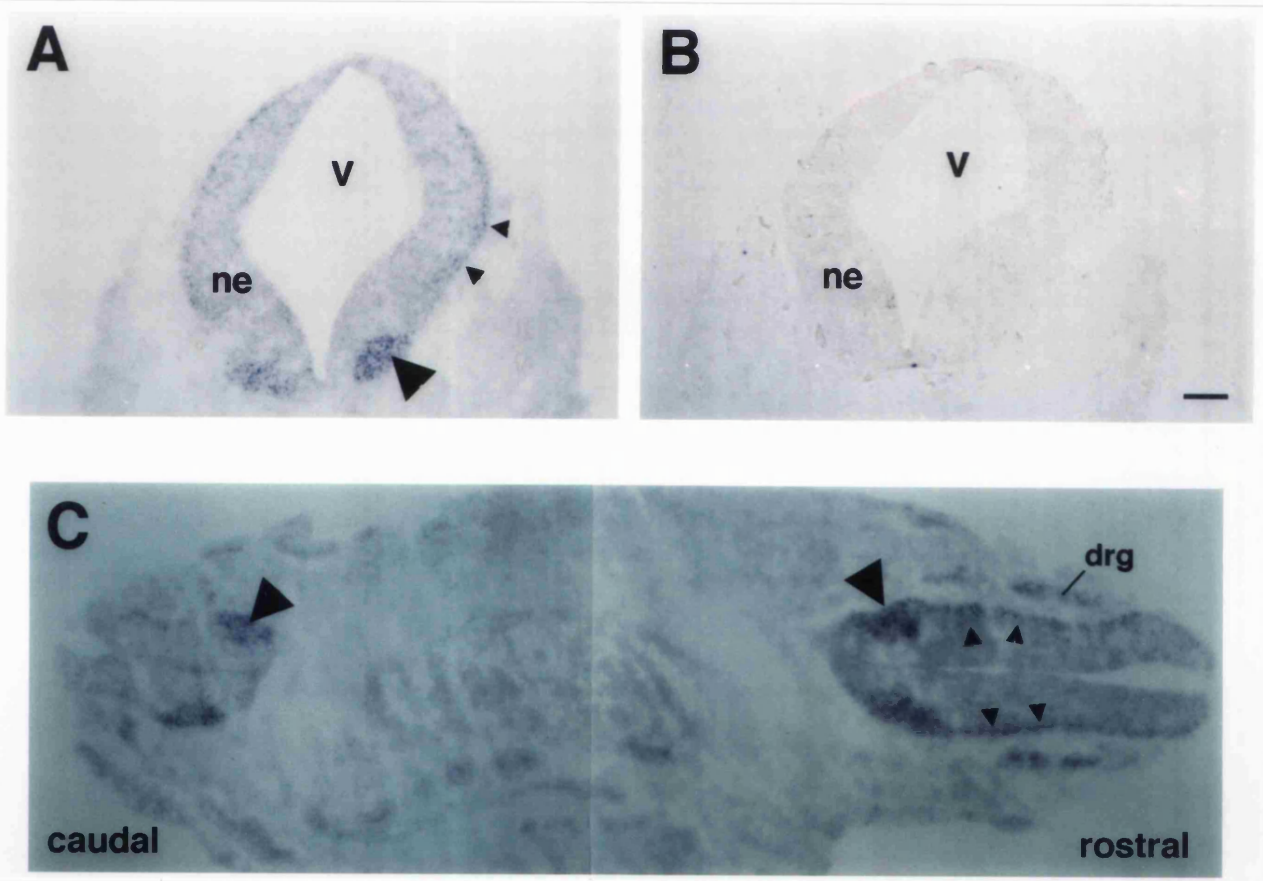


Figure 24: In situ hybridisation analysis of *Limk1* expression in the neural tube and spinal cord of E11.5 embryos. Frozen sections were hybridised with either an antisense (A) or sense (B) 2kb cRNA probe containing the C-terminal end of Limk1. The probes were labelled with DIG-UTP and visualised using anti-DIG alkaline phosphatase conjugated antibody. (**A**) Transverse section of caudal hindbrain. Regions of increased *Limk1* expression in ventrolateral regions (large arrow) and near the basement membrane (small arrows) of neural tube are indicated. ne: neuroepithelium, v: fourth ventricle. (**B**) Sense control to panel A. (**C**) Composite image of transverse section through rostral and caudal region of E11.5 embryo. Large arrows: ventrolateral expression of *Limk1* in spinal cord, small arrows: expression along basement membrane of spinal cord, drg: dorsal root ganglion. Scale bar:100μm

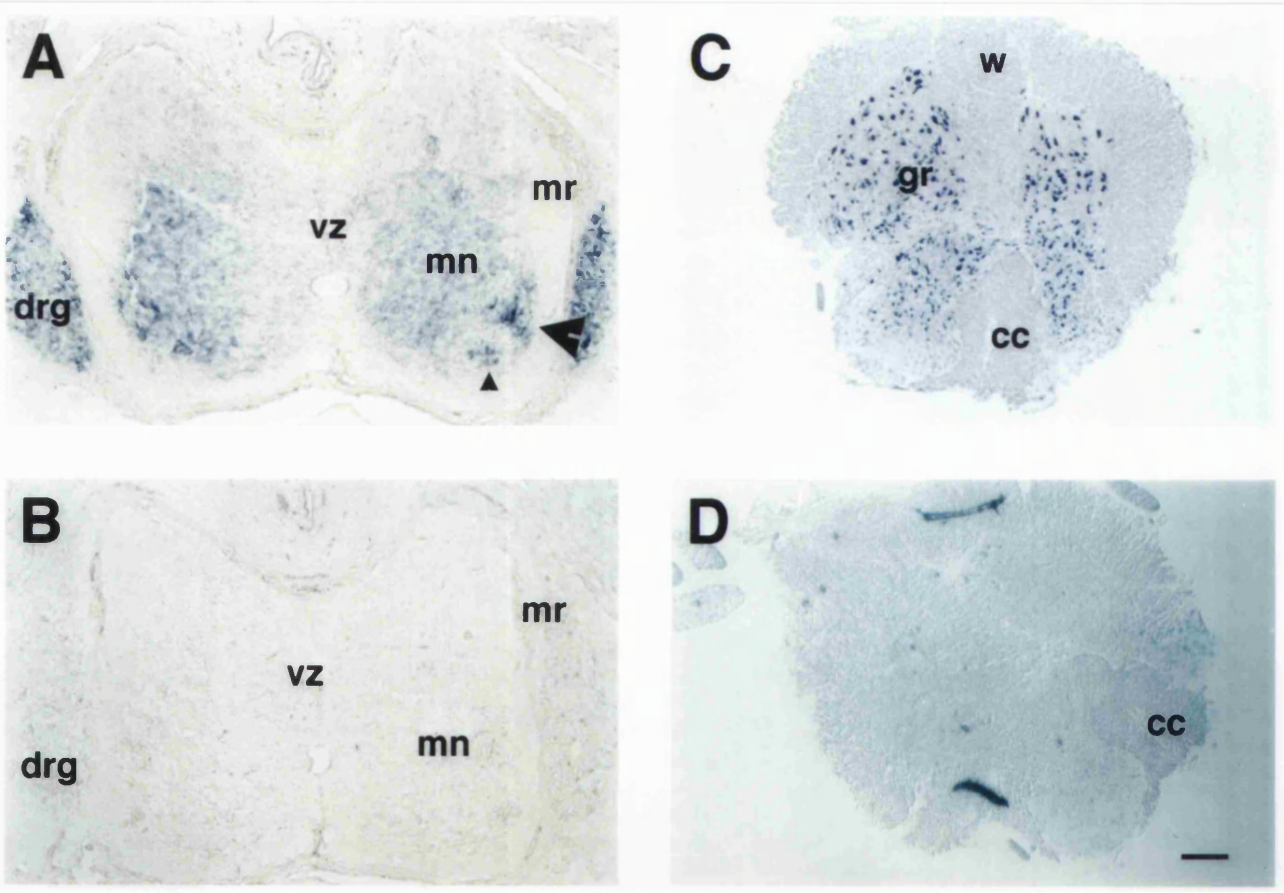


Figure 25: In situ hybridisation analysis of *Limk1* expression in the spinal cord of E14.5 embryos and adult mouse. Frozen sections were hybridised with either an antisense (A,C) or sense (B, D) 2kb cRNA probe. (**A**) Transverse section of E14.5 spinal cord at the brachial level. Arrows: regions with elevated levels of *Limk1* expression in the ventral horns, drg: dorsal root ganglion, mn: mantle layer, mr: marginal layer, vz: ventricular zone. Areas with elevated levels of *Limk1* expression are indicated by arrows. (**B**) Sense control to panel A. (**C**) Transverse section of adult spinal cord at the cervical level. cc: central canal, gr: grey matter , w: white matter. (**D**) Sense control to panel C. Scale bar:100 μm (A, B), 250 μm (C, D).

In the developing brain, *Limk1* was not only found to be expressed at various levels but also in many different regions, including structures derived from both the alar and basal plates of the neural tube. The examples shown here include the neocortex (Figures 26 and 27), the olfactory bulb (Figure 28) and the neural retina (Figure 29).

As already indicated in Figure 23C, expression of *Limk1* at day 10.5 p.c. in the forebrain vesicle is not restricted to the ventral half of the prospective prosencephalon. At day 11.5 p.c. *Limk1* appears to be evenly expressed throughout the neuroepithelium (Figure 26A). By day 12.5 p.c. regions of elevated *Limk1* expression appear in the subventricular zones of the forbrain roof (e.g. neocortex and hippocampus, Figure 26C), while *Limk1* is still expressed at lower levels in the ventricular layer. Although less pronounced, this distribution pattern of *Limk1* mRNA was also detected at later stages of embryogenesis (Figure 27A). Finally, in the adult cerebral cortex *Limk1* expression appears to be maintained in what is now the differentiating field of the neocortex, while a higher frequency of cells expressing *Limk1* can still be seen in certain layers of the neocortex (Figure 27C).

In the olfactory bulb of mid-gestation embryos, *Limk1* was also found to be expressed in the neuroepithelium, underlying the luminal epithelium (Figure 28A). Similar to *Limk1* expression in the spinal cord and neocortex, expression of *Limk1* persisted postnataly in the receptor neuron layer of the olfactory epithelium (Figure 28C).

Another example of *Limk1* expression in structures of the central nervous system is the expression observed in the neural retina of the developing eye. At day 14.5 p.c. *Limk1* message could be detected in the inner nuclear layer of the retina (Figure 29A). Although expression of *Limk1* persists in the adult eye, it is limited to the inner ganglion cell layer of the retina (Figure 29C).

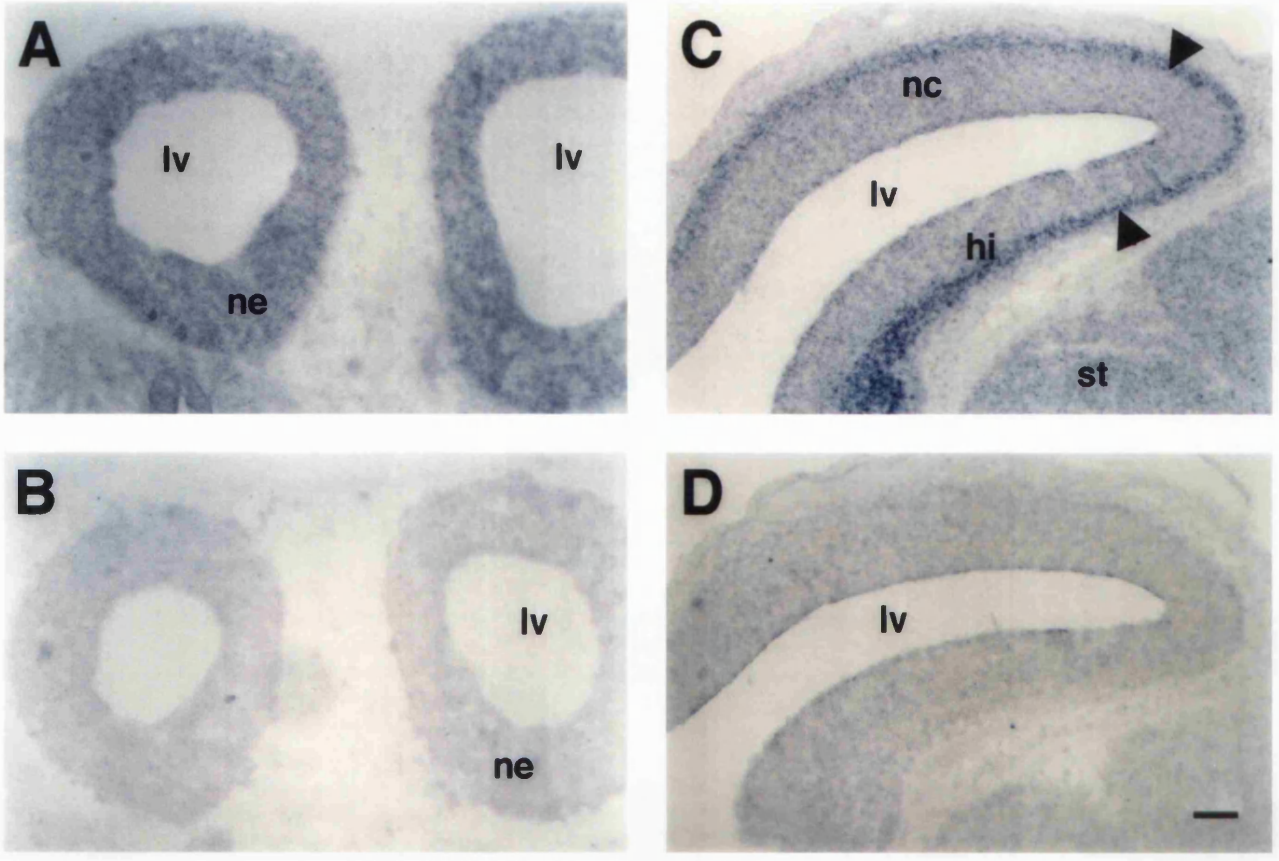


Figure 26: In situ hybridisation analysis of *Limk1* expression in the developing forebrain of E11.5 and E12.5 embryos. Frozen sections were hybridised with either an antisense (A,C) or sense (B, D) 2kb cRNA probe. (**A**) Transverse section of E11.5 telencephalic vesicle. lv: lateral ventricles, ne: neuroepithelium. (**B**) Sense control to panel A. (**C**) Sagittal section of E12.5 forebrain roof. nc: neocortex, hi: hippocampus, st: subventricular zone of striatum, lv: lateral ventricle, arrows: regions with elevated levels of *Limk1* expression in subventricular zones. (**D**) Sense control to panel C. Scale bar:100 μm.

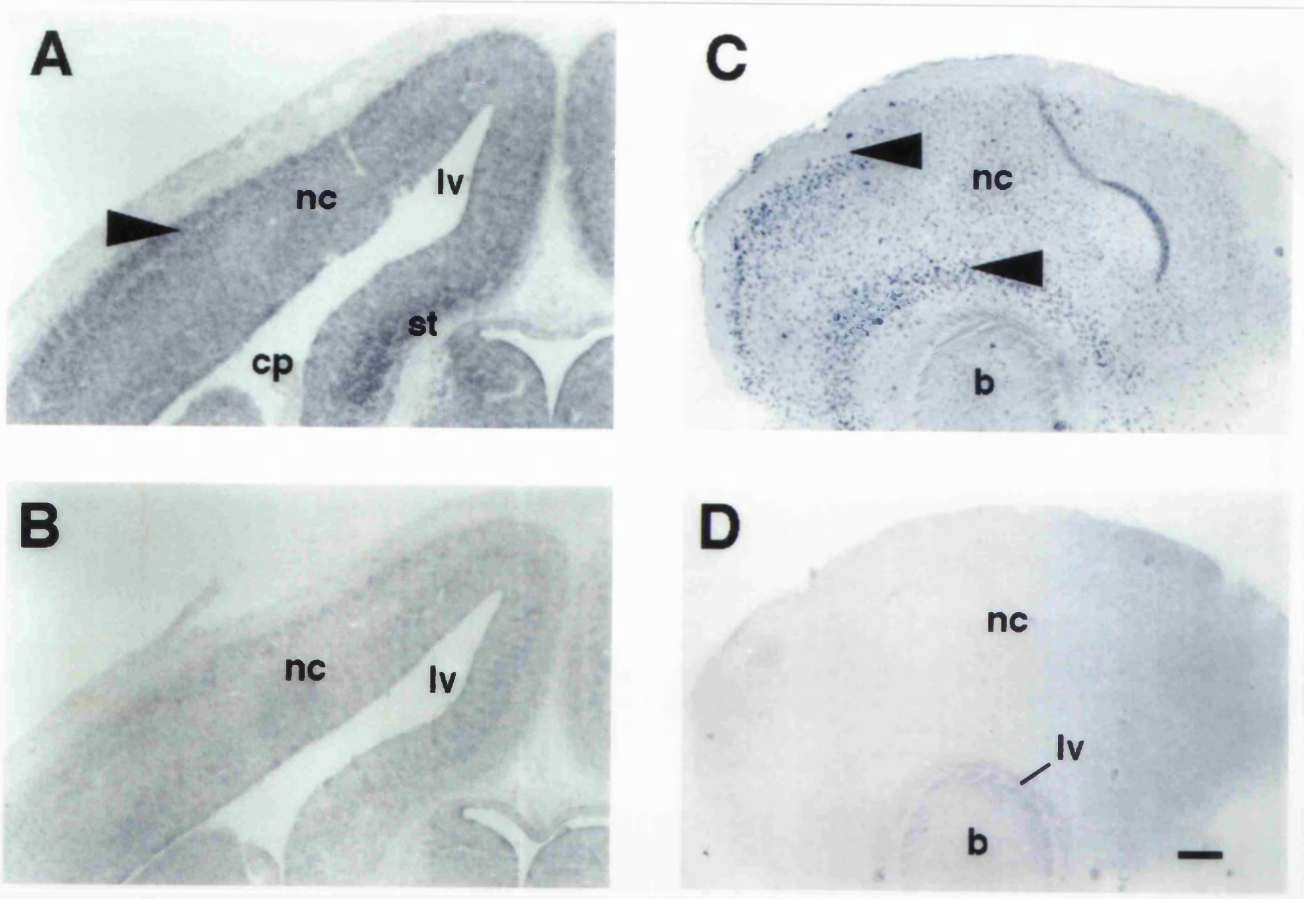


Figure 27: In situ hybridisation analysis of *Limk1* expression in the forebrain of E14.5 embryos and adult mouse. Frozen sections were hybridised with either an antisense (A,C) or sense (B, D) 2kb cRNA probe. (**A**) Transverse section of E14.5 telencephalic vesicle. lv: anterior horn of lateral ventricle, nc: neocortex, st: striatum, cp: choroid plexus, arrows: regions with elevated levels of *Limk1* expression in subventricular zones. (**B**) Sense control to panel A. (**C**) Para-sagittal section of adult neocortex. nc: neocortex, lv: lateral ventricle with fiber tracts, b: basal ganglia. (**D**) Sense control to panel C. Scale bar:100 μm (A, B), 250μm (C, D).

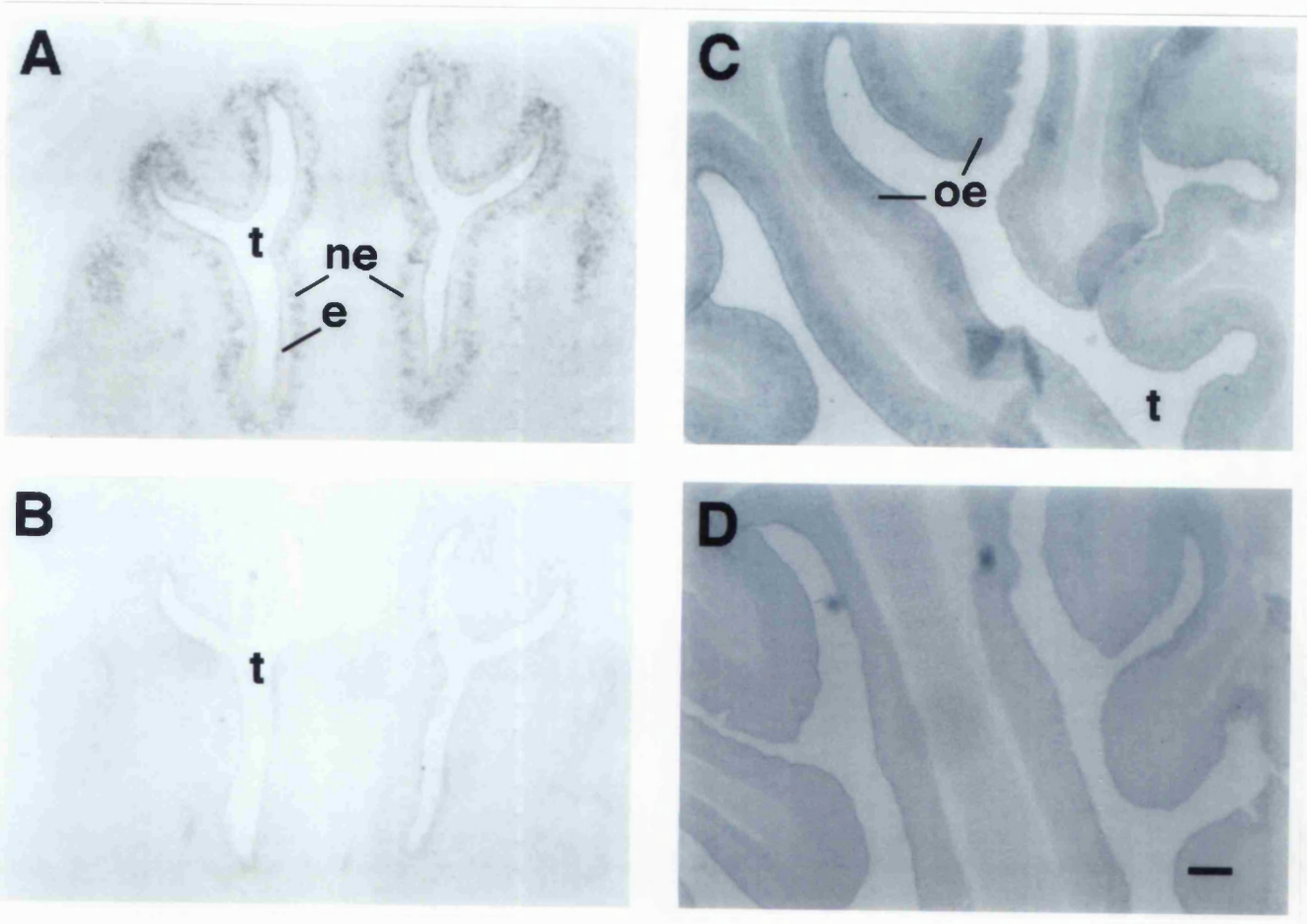


Figure 28: In situ hybridisation analysis of Limk1 expression in the olfactory epithelium. Frozen sections were hybridised with either an antisense (A,C) or sense (B, D) 2kb cRNA probe. (**A**) Transverse section of the lower head region in E14.5 embryo. t: turbinate of nasal cavity, e: epithelial sheet, ne: olfactory neuroepithelium. (**B**) Sense control to panel A. (**C**) Transverse section of the nasal epithelium in post natal day 5 mouse. oe: neural olfactory epithelium (**D**) Control to panel C. Scale bar:100 μm.

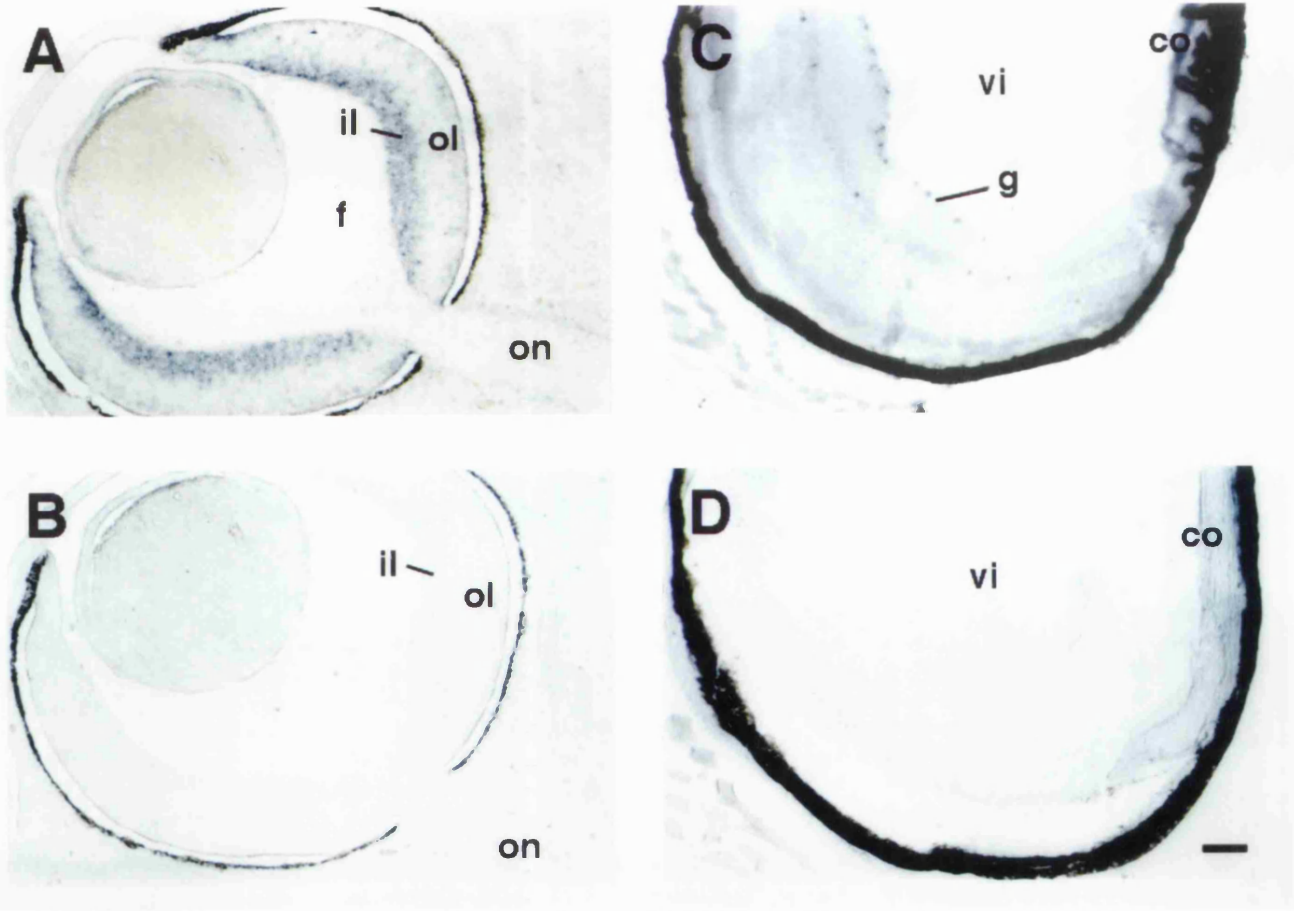


Figure 29: In situ hybridisation analysis of *Limk1* expression in the neural retina of the developing eye. Frozen sections were hybridised with either an antisense (A,C) or sense (B, D) 2kb cRNA probe. (**A**) Transverse section of the lower head region of an E14.5 embryo. f: nerve fiber layer, il: inner nuclear layer , ol: outer nuclear layer, on: optic nerve. (**B**) Sense control to panel A. (**C**) Sagittal section of adult eye. vi: vitrous humour, co: cornea, g: inner, ganglion cell layer. (**D**) Sense control to panel C. Scale bar:100µm (A, B) 250 µm (C, D).

Finally, as already indicated by the staining of the dorsal root ganglia in Figures 24 and 25, high levels of *Limk1* expression were detected in areas of the peripheral nervous system (PNS) as well as in the CNS. In fact *Limk1* was strongly expressed in all the cranial nerve ganglia (e.g. glossopharyngeal IX and vestibulocochlear VIII ganlgion, Figure 30A), the dorsal root ganglia and in the ganglia of the sympathetic nervous system (data not shown). For all cranial ganglia investigated (trigeminal V, facial VII, cochlear VIII and glossopharyngeal IX) this high level of expression persisted postnataly (e.g. trigeminal ganlion V, Figure 30B).

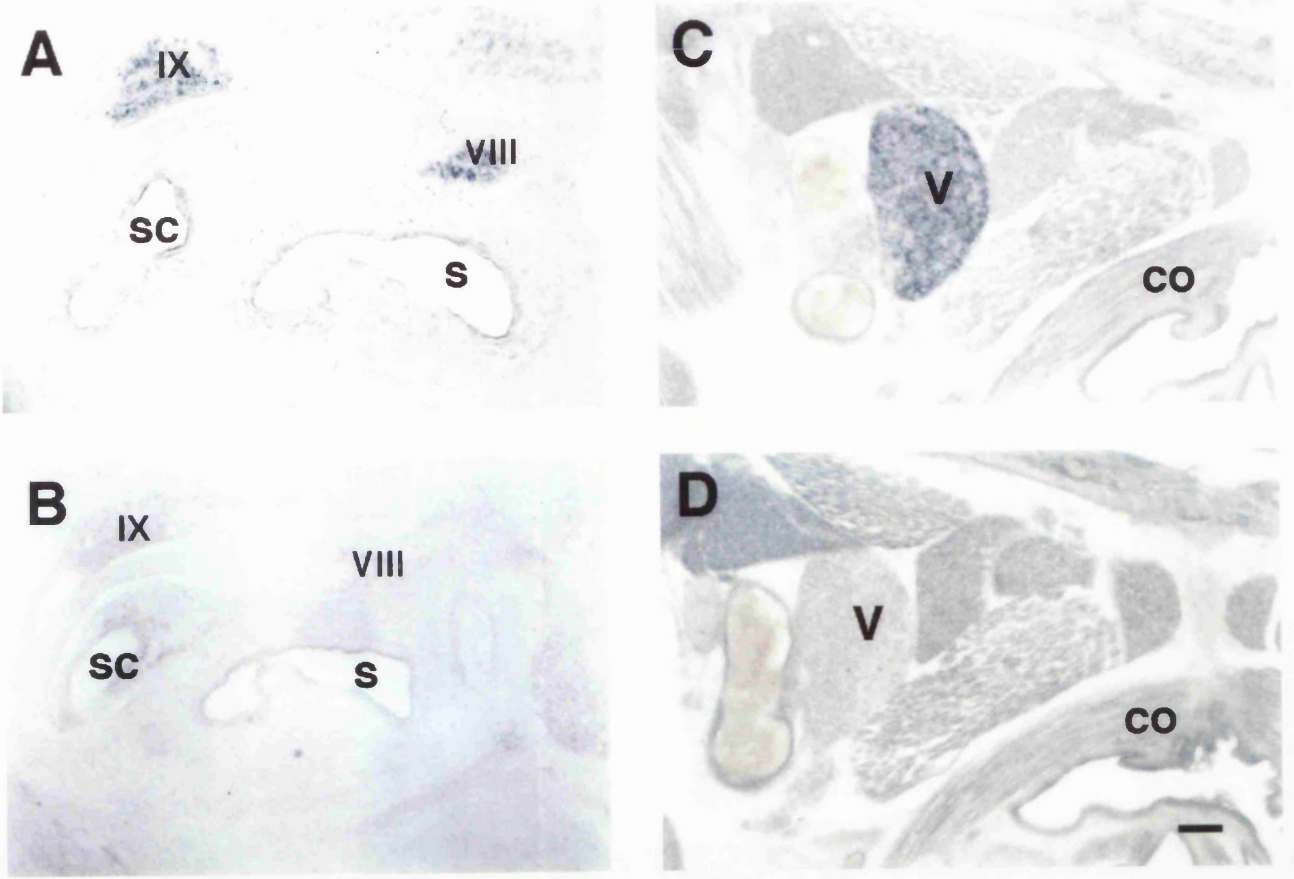


Figure 30: In situ hybridisation analysis of *Limk1* expression in cranial nerve ganglia. Frozen sections were hybridised with either an antisense (A,C) or sense (B, D) 2kb cRNA probe. (**A**) Transverse section of the lower head region of an E14.5 embryo. IX: glossopharyngeal ganglion, VIII: vestibulocochlear ganglion, sc: semicircular canal, s: saccule (**B**) Sense control to panel A. (**C**) Transverse section of postnatal day 5 head region. V: trigeminal ganglion, co: cochlea. (**D**) Sense control to panel C. Scale bar:100μm

4

Discussion

4.1 Cloning of a novel protein kinase

The study presented here describes the cloning and preliminary characterisation of the LIM-domain containing, neural kinase, *Limk*1. Fragments of the *Limk1* cDNA were initially isolated on the basis of the highly conserved peptide sequences present in the catalytic domain of all known protein kinases (Hanks *et al.* 1988; Hanks and Quinn 1991). At the time of cloning, comparison of nucleotide and predicted amino acid sequence of *Limk1* to sequences contained in the GenEMBL, NIMR and SwissProt databases, revealed that *Limk1* encoded a novel, protein kinase-like gene. Since then, Mizuno *et al.* have reported the cloning of a human cDNA sequence, LIMK, which shows a high amino acid homology to *Limk1* (Mizuno *et al.* 1994)

4.2 The catalytic domain of Limk1 defies protein kinase classification

Limk1 represents not only a novel kinase-like gene, but the combination of LIM and catalytic kinase motifs also defines a new *type* of protein kinase. In addition, the amino acid sequence of the catalytic kinase domain exhibits

unusual features which place Limk1 and LIMK into a group apart from serine/threonine or tyrosine protein kinases altogether (Figures 10 and 11). Limk1 was identified on the basis of the highly conserved amino acids found in the kinase domain of all protein kinases (see Figure 12). Indeed, all of these highly conserved residues are present in Limk1. However, residues previously thought to be diagnostic of either protein serine/threonine or tyrosine kinases in subdomains VIb and VIII deviate from either PTK or PSK consensus in Limk1. Nor does the Limk1 kinase domain show any higher degree of homology to the small and heterogenous group of dual specificity kinases (Lindberg *et al.* 1992), than to the serine/threonine or tyrosine kinases.

In light of the unusual amino acid sequence of the Limk1 kinase domain, it was necessary to test whether Limk1 encoded an active protein kinase in the first place. To test this, the Limk1 kinase domain was expressed as a glutathione transferase fusion protein. In these experiments the presence of the glutathione-S-transferase moeity served two purposes: one to allow the rapid purification of the Limk1 encoded polypeptides by the use of glutathione coupled beads, and secondly to serve as a tag with which the fusion protein could be identified on western blots. A third possible role was to artificially dimerise Limk1 proteins and thereby facilitate activation of the kinase domain, a process common to most receptor-type kinases (Schlessinger 1988; Fantl *et al.* 1993). Previously, the LIM domains of isl1 (Li *et al.* 1991; Sanchez-Garcia *et al.* 1993) have been shown to inhibit the DNA binding activity of the homeodomain. Similarly the LIM domains of Xlim-1 and mec-3 were shown to act as negative regulators (Xue *et al.* 1993; Taira *et al.* 1994). In analogy, to avoid any possible negative interference by the LIM-domains on Limk1 kinase activity, the Limk1 fusion protein used here was truncated at the amino terminus to remove the LIM-domains (Figure 13). In the presence of ^{32}P γ-ATP and either Mg^{2+} or Mn^{2+} ions,

affinity purified GST-Limk1KD fusion protein was phosphorylated (Figure 14B).

Protein phosphotransferases have been reported in prokaryotes (Hunter 1991), and to exclude the possibility that the phosphorylation of the fusion protein was due to contaminating kinases, a kinase negative mutant of Limk1 was tested in the same conditions. As seen previously for tyrosine kinases (Moran *et al.* 1988; Reith *et al.* 1991), the kinase activity of Limk1 could be eliminated by mutating the proposed catalytic D460 to asparagine (Figure 12 and Figure 15A). This result suggested that the phosphorylation of the GST-LimkKD fusion protein was due to the Limk1 kinase activity. Therefore, despite the unusual amino acid sequence of the kinase domain, the GST-Limk1KD fusion protein is capable of autophosphorylating and kinase activity can be activated *in vitro* . However, this does not resolve which part of the fusion protein is phosphorylated, and whether Limk1 auto-phosphorylates *in vivo*. Future experiments involving phosphopeptide mapping in combination with the expression of wild-type and kinase negative forms of Limk1 in suitable cell culture systems as well as the availability of a Limk1 specific antibody will be required to address this question in full (see section 4.9 below).

Initial studies using anti-phosphotyrosine western blots of the GST-LimkKD fusion protein expressed in baculovirus, revealed that the fusion protein was tyrosine phosphorylated in Sf9 cells. However, phosphoamino acid analysis of *in vitro* autophosphorylated GST-Limk1KD$^+$ fusion protein showed high levels of phosphorylation on serine residues, followed by tyrosine and threonine (Figure 16). This pattern suggests a possible dual specificity for the Limk1 kinase activity. However, hydroxy-amino acid specificity may differ between auto- and substrate phosphorylation (Lindberg *et al.* 1992). An example of this has been reported for MCK1, a protein kinase involved in the sporulation of *Saccheromyces cerevisiae* (Lim *et al.*

1993). In the absence of exogenous substrate, MCK1 was able to autophosphorylate on both tyrosine and serine residues. However, exogenous substrates are only phosphorylated by MCK1 on serine and threonine residues. Preliminary experiments using acid-denatured enolase, under various conditions, failed to show any phosphorylation of exogenous subtrate by the GST-Limk1KD fusion proteins. As the *in vitro* kinase assay conditions used here are capable of supporting autophosphorylation, it is possible that enolase is an unsuitable substrate for Limk1 kinase. In addition, the use of exogenous subtrates which are not the physiological substrates of Limk1 may still not reflect the *in vivo* phosphoamino acid specificity of Limk1. Such a discrepancy between *in vitro* kinase activity and *in vivo* subtrate phosphorylation has been observed for ERK1/MAP kinase. While ERK1/MAPK was shown auto phosphorylate on tyrosine and threonine residues *in vitro* (Seger *et al.* 1991), all known *in vivo* substratesof MAP kinase have only been been found to be phosphorylated on serine and threonine residues. In addition the primary sequence of substrates for the ERK/MAP kinases 1 and 2 has been determined to be Pro-x_{1-2}-$^{Ser}/_{Thr}$-Pro, where x denotes a neutral or basic amino acid (Alvarez *et al.* 1991; Gonzalez *et al.* 1991).

In addition, hydroxy-amino acid specificity of Limk1 kinase activity may be regulated by the presence or absence of certain divalic metal ions. Routinely, Mg^{2+} ions are added to *in vitro* kinase reactions. This co-factor facillitates the binding of the tri-phosphate moeity of ATP to the nucleotide binding site of protein kinases (Taylor and Adams 1992). One example of how this cofactor can affect hydroxy-amino acid specificity comes from phosphorylase kinase (Yuan *et al.* 1993). In the presence of Mg^{2+} ions this enzyme will serine-phosphorylate phosphorylase *b*, but Mn^{2+} activates tyrosine phosphorylation of angiotensin II. Limk1 protein kinase is active in the presence of either divalic ion (Figure 14) and addition of both Mg^{2+} an

Mn2+ ions leads to an additive effect on the autophosphorylation of the GST-Limk1KD fusion protein. It will be interesting to test whether the hydroxy-amino acid specificity of Limk1 can also be regulated by the presence of specific metal ions.

4.3 *Limk1* is also a new member of the LIM-domain protein family

By combining two LIM-domains in the amino-terminal half and a protein kinase domain in the carboxy-terminal half of the molecule, Limk1, together with the human LIMK protein (Mizuno *et al.* 1994), defines a new type of LIM-domain protein (Figures 3 and 8), distinct from the LIM-homeodomain, LIM-only and Multi-LIM domain proteins (Sanchez-Garcia and Rabbitts 1994). However, like the LIM-homeodomain proteins, the LIM domains are located at the amino--terminal, while the second effector domain is located towards the carboxy-terminal end of the protein. This arrangement is different from the corboxy-terminal LIM domains of paxillin and zyxin, while the LIM-only proteins consist almost entirely of LIM-domains.

Phylogenetic analysis of the majority of known LIM-domain proteins reveals that these can be roughly subdivided into three major groups: the group containing all cysteine-rich proteins (CRP), the group of LIM-homeo-domain proteins, and sandwiched in between, members of the Multi-LIM class of proteins. (Figure 9) Limk1 and LIMK cluster with the group of nuclear, homeodomain containing LIM-proteins. Interestingly the only other LIM-protein found in this class and which does not contain a homeodomain is RBTN1. RBTN2 and 3 have not been included in the phylogentic tree, but are likely to map to the same region on the basis of their very high homology to RBTN-1 (Foroni *et al.* 1992). To date the Rhombotin group of proteins are the only LIM-proteins which have been shown to act as nuclear oncogenes

(Fisch *et al.* 1992). Rhombotins and LIM-homeodomain proteins are both nuclear proteins, and as discussed below, *Limk1* and LIMK both contain a putative nuclear localisation signal. However, it is unlikely that the LIM domains of all nuclear proteins share a recent precursor, as the LIM domains of another nuclear protein, MLP, are closer related to the group of cytoplasmic cysteine-rich proteins (CRP). The symmetrical distribution of the first (L1) and second (L2) LIM domains in proteins with two LIM domains, suggests that the tandem array of LIM domains has arisen by an intragenic duplication within a common ancestorial LIM protein. The clear distinction between the CRP group of LIM proteins and the LIM-homeodomain group, suggests at least one further gene duplication before the ancestorial genes of these two groups diverged. As the phylogenetic tree in figure 9 is unrooted, the zinc-finger-like regions of protein kinases C α and ζ have been included as an example for the only other protein kinase known to contain a zinc finger-like motif. Clearly, the zinc finger regions of *Limk1* and protein kinase C are only very distantly related.

4.4 The LIM-domain is a multifunctional domain with as yet unpredictable consequences for Limk1

While it is clear that protein kinases affect numerous cellular functions through their ability to phosphorylate target proteins (see section 1.2), the function of LIM domains is less clear. Indeed, LIM domains may possess multiple functions (see section 1.3). Recently the three-dimensional structure of the carboxy-terminal LIM domain of CRIP has been determined by NMR spectroscopy (Perez-Alvarado *et al.* 1994). From this study it has been concluded that the LIM/double zinc-finger motif closely resembles the GATA1-type zinc finger (Sanchez-Garcia and Rabbitts 1994). This supports

the hypothesis that LIM domains may be involved in binding to DNA (Freyd *et al.* 1990; Karlsson *et al.* 1990). However, no DNA binding activity has yet been described for LIM-domains, and LIM-proteins which have been shown to bind DNA do so through their homeodomain. In fact in Isl1 the LIM-domains act to inhibit binding to target DNA sequences (Karlsson *et al.* 1990; Xue *et al.* 1993). Although the LIM-domains of Isl-1, when fused to the homeodomain of Ultrabithorax, were able to inhibit DNA binding activity of this heterologous homeodomain, it is still unclear whether the inhibitory effect is the result of *intra-* or *inter*-molecular interactions (Sanchez-Garcia *et al.* 1993).

LIM domains can also mediate stimulatory effects. The LIM-domains of Lmx-1 are required for synergistic activation of the insulin mini-enhancer by the cooperation of Lmx-1 and Pan-1, a helix-loop-helix protein (German *et al.* 1992). Similarly, the myogenesis promoting activity of MLP is dependent on the presence of the LIM-domains (Arber *et al.* 1994). But while a number of helix-loop-helix proteins are involved in muscle differentiation, MLP has not yet been shown to interact directly with any of these. The direct interaction of LIM domains and helix-loop-helix proteins has recently been demonstrated for RBTN1 and 2. Both are capable of binding to the helix-loop-helix domain of TAL-1, 2 and LYL1 in myeloid cells (Valge-Archer *et al.* 1994; Wadman *et al.* 1994). It will be interesting to see what effect this has on the functions of TAL-1, 2 and LYL1. Finally, the interaction between the LIM domain of P-Lim and the POU-domain of Pit-1 has recently been shown to be a requirement for the synergistic activation of the prolactin promoter (Bach *et al.* 1995). Taken together with the ability of zyxin to bind cCRP via the LIM domains (Schmeichel and Beckerle 1994), this clearly demonstrates a role for LIM domains in protein-protein interactions and suggests that these interactions may inhibit or activate associated protein modules.

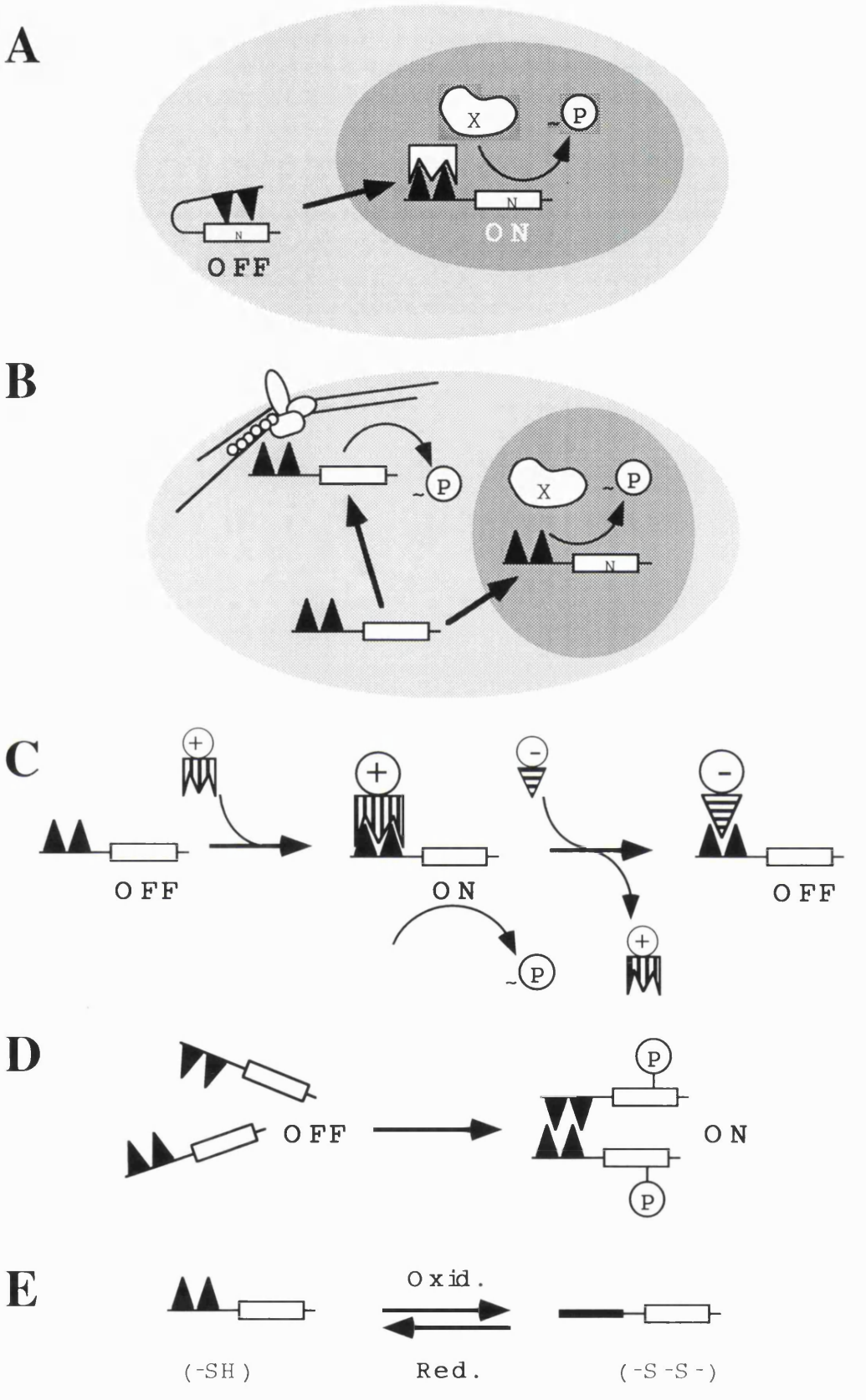
By analogy, the role of the LIM-domains in Limk1 may be highly diverse. The LIM-domains may act through an intramolecular mechanism to mask the putative nuclear localisation signal in a manner analogous to the binding of the ankyrin-like repeats to the Rel region of p105 NFκB (Blank *et al.* 1992), thus controlling a possible nuclear localisation of Limk1 (Figure A). Alternatively, Limk1 may be targeted to specific cell compartments, such as the cytoskeleton or the nucleus, through direct binding of the LIM domains to other proteins or even DNA (Figure B). However, the subcellular localisation of Limk1 may also be independently regulated by either the putative nuclear localisation signal located within the kinase domain insert, or the DHR motif located in the intervening domain of Limk1. The DHR motif was originally identified in a class of intracellular proteins associated with signal transduction at cell junctions (Koonin *et al.* 1992). Many DHR-motif containing proteins localise to the cell membrane and *in vitro* experiments have recently demonstrated the ability of the DHR domain of HDLG to bind to the amino-terminal domain of protein 4.1, itself a cytoskeletal protein (Lue *et al.* 1994). Therefore, the putative DHR-motif in Limk1 may play a role in targeting Limk1 to cytoskeletal structures at the cell membrane. Indeed, substrate specificity may arise in part through the localisation of protein kinases and their substrates to common sites (Hunter 1994). Likewise, subcellular localisation will undoubtedly affect substrate availability and therefore the specificity of Limk1 kinase. At the same time different cellular compartments will expose Limk1 itself to different regulatory signals (see below).

In addition, LIM domains may directly effect the activity of the kinase domain by binding inhibitors or activators of Limk1 (Figure C, Schmeichel and Beckerle 1994) or mediate the formation of Limk1 multimers in a manner analogous to receptor kinases (Figure D, Schlessinger and Ullrich 1992). Such a model can be extended to include the direct recruitment of

Limk1 kinase substrates by the LIM domains. As domain swapping and deletion experiments have shown, single LIM domains are capable of functioning independently of neighbouring LIM domains (Schmeichel and Beckerle 1994). It is therefore possible for the two LIM domains in Limk1 to perform separate functions.

One further level of control which may be mediated through the LIM domains comes a result of the high cysteine content of LIM domains. Like all zinc-finger like domains, this makes them particularly sensitive to the redox state within the cell. A drop in the redox state could lead to the formation of disulfide bridges, thereby disrupting the tetrahedral binding of the zinc ions (Figure E). The only example of redox regulation comes from studies involving the homeodomain of isl-1 (Sanchez-Garcia and Rabbitts 1993). However, in the absence of a homeodomain in Limk1, this type of control may be mediated by the LIM domains of Limk1.

Figure 31: Schematic presentation of the possible effects the LIM domains may have on the function of Limk1. (**A**) The LIM domains interact in an intramolecular way to mask the putative nuclear localisation signal. Following activation, by covalent modification or the binding of a second protein, the nuclear localisation signal becomes unmasked and active Limk1 is translocated to the nucleus. (**B**) The LIM domains serve to target Limk1 to different subcellular compartments, including the cytoskeleton, cytoplasm or nucleus. (**C**) LIM domains act as receptors for activators or inhibitors of the kinase activity (Schmeichel and Beckerle 1994) (**D**) LIM domains mediate dimerisation of Limk1 molecules, which leads to cross-phosphorylation and activation of Limk1 (Schlessinger and Ullrich 1992) (**E**) LIM domains act as kinase subtrate binding surface.


A
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(-SH)
(-S-S-)

4.5 Limk1 itself may also be a target for other protein kinases

Irrespective of the effect that the LIM domains may have, Limk1 itself may also be regulated through phosphorylation by other kinases involved in signal transduction. Putative recognition motifs for casein kinase I and MAP kinase and protein kinase C lie within the proline/serine-rich region connecting the LIM and kinase domain. Although little is known about Casein kinase I, both MAP kinase and protein kinase C are key regulatory molecules of overlapping pathways. This suggests Limk1 may be at the receiving end of the pp74raf/MAP kinase pathway, which is implicated in mediating the immediate early response growth reponse (reviewed in Cano and Mahadevan 1995; Marshall 1995). Numerous examples exist of the stimulatory and inhibitory effects of phosphorylation by MAP kinase. These include the activation of transcription factors like Elk-1 (Hill *et al.* 1993; Marais *et al.* 1993), c-myc (Seth *et al.* 1991), or pointed (Brunner *et al.* 1994), or the activation of cytoplasmic phopholipaseA2 (Lin *et al.* 1993), or the protein kinases MAPKAP-2 (Stokoe *et al.* 1992) or RSK (Erikson 1991). However, examples of inhibitory effects of MAP kinase have also been described, like the inhibition of the inhibitory transcriptional regulator, yan, in *D. melanogaster* (Brunner *et al.* 1994; Brunner *et al.* 1994). An example of both positive and negative regulation by phosphorylation of transcription factors comes from c-jun. While the amino-terminal phosphorylation of jun results in activation of jun (Pulverer *et al.* 1991), phosphorylation of the carboxy-terminus leads to inhibition of DNA binding by jun protein present in AP-1 (Boyle *et al.* 1991). It has recently been demonstrated that the stress related phosphorylation which leads to the activation of c-jun is carried out by JNK/SAPK1 and 2, also members of the MAP kinase subfamily (Smeal *et*

al. 1991; Hibi *et al.* 1993). The substrate recognition sites for these MAP kinases have not yet been determined. Nonetheless, it is important to realise that phosphorylation by MAP kinases may have completely opposite effects, making it difficult to predict what effect MAP kinase activation may have on Limk1. At this point it should be noted that although Limk1 may be post-translationally modified by either of these kinases, the expression of Limk1 is not increased in starved NIH 3T3 cells following stimulation with serum (data not shown). Therefore unlike the cysteine-rich proteins, Limk1 itself does not respond as an immediate early gene (Wang *et al.* 1992; McLaughlin *et al.* 1994).

In addition to phosphorylation on serine or threonine residues, Limk1 contains several tyrosine residues which may be phosphorylated. Although none of these match perfectly to known SH2 binding sites (Songyang *et al.* 1993), one carboxy-terminal tyrosine residue (Y630) may represent a binding site for an as yet unidentified src-type SH2 domain. Such domains prefer hydrophilic amino acids at the +1 and +2 positions and have a small hydrophobic pocket which accomodates a hydrophobic residue at position +3 (Pawson 1995).

4.6 The *Limk1* expression pattern: a possible clue to *in vivo* function?

Northern blot analysis of *Limk1* expression reveals a predominant expression of *Limk1* in cells and tissues which are derived from the nervous system (Figures 20, 21 and 22). Already at day 9.5 p.c. in the mouse embryo, high levels of a 3.5kb *Limk1* mRNA can be detected in the neuroepithelium. These high levels of expression are maintained in the adult spinal cord. Expression of *Limk1* in purified cultures of rat glial cells (oligodendrocytes,

Schwann cells, oligodendrocyte-type 2 astrocyte precursors and type-1 astrocytes) suggests that *Limk1* may be expressed by cells of the glial compartment *in vivo*. Particularly interesting is the expression of *Limk1* in both oligodendrocytes and their precorsor cells, the O-2A progenitor (Richardson *et al.* 1990). Whether the increase of *Limk1* message in oligodendrocytes plays any role in the differentiation of O-2A progenitor cells into oligodendrocytes remains unclear at present. However, the O-2A lineage culture system will be invaluable in addressing this question (see section 4.9). Finally, expression of *Limk1* in F1 melanoma cells points towards the expression of *Limk1* in lineages derived from the neural crest.

Like LIMK, the initial cDNA fragment of *Limk1* was also isolated from a liver cell line (HepG2 for LIMK, tsA58-L25 for *Limk1*). In the embryonic day 13 p.c. liver cell line tsA58-L25, *Limk1* was expressed at relatively low levels. Surprisingly, *Limk1* mRNA was not found in either embryonic or adult mouse liver. Instead, *Limk1* was expressed in cultured meningeal cells and NIH 3T3 fibroblasts (data not shown). Both of these connective tissue cell types are representatives of the mesodermal compartment. However, *Limk1* was apparently not detected in the connective tissue of other organs, such as heart, kidney, liver, intestine, spleen or muscle. Nor did *in situ* hybridisation analysis detect *Limk1* mRNA in meningeal cells of the adult brain (see below and Figures 27 and 28). The fact that *Limk1* is expressed in cultured cells derived from tissues which apparently do not express *Limk1 in vivo*, may be due to several different reasons: (a) *Limk1* is expressed in these cell types *in vivo* at a level below the detection limit of Northern blot or *in situ* hybridisation analysis, and is subsequently upregulated *in vitro*. (b) *Limk1* is expressed at higher levels in only a very small population of cells within the tissue and these cells gave rise to the bulk of the cell culture tested here for *Limk1* expression. Finally, (c) *Limk1* may not normally be expressed in the tissue analysed, but is expressed ectopically *in vitro*. The later suggests

that *Limk1* may confer some property to cells which promotes their survival *in vitro* thereby selecting for *Limk1* expressing cells.

A recurring theme appears to be the role of LIM-domain proteins in the differentiation of specific cell-types, in particular neural cells. Among the vertebrate LIM proteins this has been postulated for: *Gsh-4* (Li *et al.* 1994), *Islet-1*, *LH-2* (Xu *et al.* 1993), *lim-1* (Fujii *et al.* 1994), *mLim-3/P-Lim*, the rhombotins 1 (Greenberg *et al.* 1990) and 2 (Warren *et al.* 1994), and Xlim-1 (Taira *et al.* 1992). A number of LIM-proteins exhibit an expression pattern which overlaps significantly with that of *Limk1*. Throughout development *Limk1* is expresssed in the spinal cord. At the earliest stages investigated, expression is restricted to the ventrolateral region of the neural tube and to the neuroepithelial cells along the basement membrane (Figure 24). These areas have been shown to be among the first regions to be populated by differentiating neuroblasts in the spinal cord (Langman and Haden 1970; Nornes and Das 1974; Placzek *et al.* 1991). In particular, motor neuroblasts, which originate from the ventral regions of the ventricular zone arise between days 11 and 13 of gestation in the rat spinal cord (roughly equivalent to days 10 to 12 in the mouse) and migrate laterally (Langman and Haden 1970). Although by day 14.5 p.c. *Limk1* was found to have spread to other regions of the mantle layer, no *Limk1* expression was detected in the proliferating ventricular zone (Figure 25A). At the same time the ventrolateral areas of the spinal cord which expressed elevated levels of *Limk1* overlapped significantly with the developing median and lateral motor columns (MMC and LMC ,Tsuchida *et al.* 1994). In fact, the intrasegmental distribution of *Limk1* resembles that of *Islet-2* at the brachial level of the chick spinal cord (Tsuchida *et al.* 1994). Together with *Islet-1* (Thor *et al.* 1991; Ericson *et al.* 1992), *Lim-1* and *Lim-3* (the chick homologue of Xlim-3, Taira *et al.* 1993) , the expression *Islet-2* in neuroblasts has recently been shown to define subclasses of motorneurons (Tsuchida *et al.* 1994). However, in the adult

Limk1 expression does not correlate with motor neuron columns and is found to be evenly distributed throughout the grey matter. Therefore it remains to be seen whether *Limk1* plays any part in the "LIM-code" which has been appears to determine the identity and axonal pathfinding of motor neuron subclasses. Interestingly, RBTN1 (Greenberg *et al.* 1990) is also expressed in the ventral horns of the developing spinal cord, and similar to *Limk1*, in the inner layer of the embryonic retina (Figure 29A). Furthermore, like *Limk1*, *LH2* (Xu *et al.* 1993) and RBTN1 (Boehm *et al.* 1991) are also expressed in cerebellum, cortex (Figures 26 and 27) and olfactory bulbs (Figure 28). However, only *Islet1* (Dong *et al.* 1991) and *Limk1* are also expressed in sympathetic ganglia. Unlike the neuroepithelium in the ventricular zones of the CNS and the dorsal and mid line regions of the spinal cord, which exhibit a high level of proliferation, the areas of *Limk1* expression represent differentiating fields with low mitotic activity and primarily populated by post-mitotic cells (Altman and Bayer 1984; Placzek *et al.* 1991). The localisation of *Limk1* to these areas and the continued expression of *Limk1* in the adult nervous system (see Figures 25C, 27C, 28C, 29C and 30C) argues for a possible role in the differentiation and the maintenance or survival of these differentiating cells. Detailed *in situ* analysis of adult tissues will be needed to identify the cells expressing *Limk1* in non-neural tissues, like the thymus, lung and reproductive organs. It should also be noted that similar to RBTN1 (Greenberg *et al.* 1990) and LH2(Xu *et al.* 1993), *Limk1* seems to be expressed in the embryo in both neuronal and glial cell types, or their precursor cells. Northern blot analysis using purified cultures of primary oligodendrocytes derived from the optic nerve (Figure 21) confirm that *Limk1* is expressed in glial cells *in vitro*. At the same time, expression of *Limk1* early on in the development of the inner nuclear layer of the retina (Figure 29A), prior to the differentiation of glial cells (Finlay and Sengelaub 1989), and the absence of *Limk1* expressing cells in the white matter of the

spinal cord suggest *Limk1* may also be expressed in neuronal cells or the precursors of neuronal and glial cell types. Finally, the expression of *Limk1* in cranial ganglia (Figure 30), dorsal root ganglia (Figures 24C and 25A), Schwann cells (Figure 22) and F1 Melanoma cells (Figure 21) suggest that *Limk1* may also play a role in derivatives of neural crest.

4.7 *Limk1* is not associated with any mapped mutant phenotypes

Additional information regarding the *in vivo* function of a novel gene can also be gained from mouse strains which carry a mutation in the gene of interest. In order to determine whether such a mutant strain existed for *Limk1*, the chromosomal location of *Limk1* was mapped. High stringency Southern blot analysis revealed that *Limk1* is a single copy gene, localised at the distal end of mouse chromosome 5 (Figure 17). Linkage analysis did not reveal any known mouse mutants which map to the viccinity of *Limk1*. *Nos1* encodes the brain nitric oxide synthase (William O'Brian, Baylor College of Medcine, unpublished observation), while *Zp3* encodes the zona pellucida protein 3 (Lunsford *et al.* 1990) and has also been mapped in the human (van Duin *et al.* 1993). *Ipf1* encodes the insulin promoter factor 1 (Leonard *et al.* 1993). *Pmv-12* represents the location for an insertion site of the polytropic leukemia virus-12 (Frankel *et al.* 1989) and *Iapls 3-9* also represents sequences of an inserted provirus (Lueders *et al.* 1993). Linkage with *Zp*3 and *Ipf1* predicts this region of mouse chromosome 5 to be syntenic with human chromosome region 7q21-22. Again no mutations or chromosomal abberations of this region have been reported in patients suffering of symptoms which may be consistent with the expression pattern of *Limk1*.

4.8 Limk1: a potential new player in signal transduction pathways

Like many other kinases which are themselves phosphorylated, Limk1 may play a role in amplifying and integrating cell signalling during normal development. In this capacity, the potential for LIM-domains to interact with other LIM-domains, other protein domains altogether (*ex*: helix-loop-helix proteins) or possibly DNA sequences may allow Limk1 to play a role in integrating cell signalling during normal development. The recent report of an oncogenic fusion between the helix-loop-helix domain of *tel* and the kinase domain of the PDGF β receptor in chronic myelomonocytic leukemia patients emphasises the potential impact which the combination of a protein binding domain, normally present in transcription factors, and a catalytic kinase domain may have on cell proliferation and differentiation (Golub *et al.* 1994; Sawyers and Denny 1994). This is particularly relevant as Dr. Nick Gutowski and Robert Ludwig in our laboratory have demostrated high levels of *Limk1* expression in a large number of primary samples and subcultured cell lines of human glioblastomas (personal communication). As *Limk1* is also expressed in primary glial cells it will be of the essence to study the function and regulation of Limk1 protein in this highly aggressive tumour type.

4.9 Outlook

With the availability of full-length cDNA and the appropriate constructs for the expression of *Limk1*, we are now in a position to begin a more detailed biochemical analysis of Limk1 protein. An important first step is the generation of Limk1-specific antibodies, which will allow us to determine the intracellular localisation of Limk1. This will also enable us to study the association of Limk1 *in vitro* with known proteins by immunoprecipitation and western blot techniques, and the isolation of novel Limk1-binding proteins by using antibody trapping protocols. In addition, the use of two-hybrid systems will be invaluable to test protein -protein interactions between the LIM domains of Limk1 and candidate binding partners. To facilitate the identification of Limk1 subtrates, screening of a degenerate peptide library may prove useful (Songyang *et al.* 1994). The generation of various mutants of Limk1 will complement this approach to determine the subtrates, binding proteins and the molecular nature of Limk1 function.

Another course of research is the use of *in vitro* cell systems to study the effects of Limk1 on cell behaviour. The fact that *Limk1* is expressed in the cells of the oligodendrocyte-type 2 astrocyte (O-2A) lineage allows us to exploit the good understanding of this cell lineage system (Noble and Murray 1984; Richardson *et al.* 1990; Groves *et al.* 1993). Specifically, over-expression of wildtype or mutant forms of *Limk1* can be tested for effects on O-2A precursor differentiation, proliferation and survival. Alternatively, the application of antisense oligonucleotides, which have been used to block proteins (both *in vivo* and in tissue culture) for which the ligand is unknown (Hunter *et al.* 1995), may allow us to rapidly dissect the function of *Limk1* in primary cells. In addition the regulation of *Limk1* can be studied using the embryonic stem cell line D3, which can be induced to differentiate *in vitro*.

Again, expression of mutated forms of *Limk1* can be used to assess the possible function of *Limk1* in this pluripotent cell line. Finally the expression of *Limk1* in PC12 cells may prove useful in addressing the possible role of *Limk1* in cells of the neural crest lineage.

In addition to the ongoing biochemical analysis, it will be imperative to study the role of Limk1 in the context of the developing organism. Our initial studies of the *Limk1* expression pattern during mouse embryogenesis has already given insights into the possible role of Limk1. A detailed analysis of the spatial and temporal regulation of both *Limk1* mRNA and protein expression will enhance our understanding of Limk1 function. However, experimental embryology involving the *in vivo* manipulation of either mouse or chick embryos is most likely to reveal how Limk1 ties in with the developmental program of the nervous system. Clearly, the production of transgenic mice which carry either a null mutation or a dominant negative mutant form of Limk1 is a priority. In addition, our ability to study the behaviour of the O-2A lineage cells *in vitro* provides us with an additional means of analysing the effect of such transgenes in great detail. O-2A progenitor cells isolated from *Limk*1 transgenic animals could then be tested with respect to their ability to proliferate and differentiate in response to a number of known growth factors. Should the total deficiency of Limk1 prove to be lethal to the organism, tissue-specific knock-outs could be generated using the Cre/*loxP* system (Gu *et al.* 1994). In such an event, the promoter of the myelin-basic protein (MBP) could target expression of Cre recombinase to the oligodendrocyte compartment.

Finally, the high levels of Limk1 expression detected in several human glioblastoma samples raises the question of whether Limk1 is involved in the initiation, growth control or tumourgenicity of this particularly agressive form of brain tumour. The availability of the human glioblastoma line 1789, which was established in our laboratory and exhibits an O-2A phenotype,

provides us with the unique opportunity to address this question *in vitro* (Noble *et al.* 1995).

While each approach promises to yield new insights into the function and regulation of this novel protein kinase, it is clear that a combined strategy will generate a more complete understanding of Limk1 function. I hope to contribute towards this exciting goal.

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