Molecular and cell biological studies of a novel intracellular rat brain ion channel protein

by

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Declaration of Originality

I declare that, unless otherwise stated, this thesis represents my own work and was composed by me

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Abbreviations	
A	absorbance
A	adenosine
A	alanine
AD	GAL 4 activation-domain
AEBSF	4-(2-aminoethyl)-benzenesulphonyl fluoride
AMV	avian myoblastoma virus
BD	GAL 4 DNA-binding domain
bp	base-pair(s)
BSA	bovine serum albumin
C	carboxy
C	cystein
C	cytosine
cAMP	cyclic adenosine 5'-phosphate
cDNA	complementary DNA
°C	degrees Celsius
cRACE	circle- or concatamer-mediated RACE PCR
D	aspartic acid
Di-O-C_5(3)	3-3'-dipentyloxacarbocyanine iodide
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
E	glutamic acid
ECL	enhanced-chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethylether) N, N, N', N'-tetraacetic acid
ER	endoplasmic reticulum
F	phenylalanine
FRB	formaldehyde running buffer
G	guanine
G	glycine
g	gravity
H	A/T/C not G
H	histidine
h	hours
His	histidine
HRP	horseradish peroxidase
I	inosine
I	isoleucine
IAA	indanyloxyacetic acid
IgG	immunoglobulin G
IPTG	isopropyl-β-thiogalactopyranoside
IP_3	inositol 1, 4, 5-Trisphosphate
IP_3R	IP_3 receptor

K	lysine
kb	kilobase-pair(s)
kDa	kiloDaltons(s)
L	leucine
l	litre
lacZ	β-galactosidase gene
LB	Luria-Bertani medium
LDPCR	long-distance PCR
Leu	leucine
λ	bacteriophage lambda
M	methionine
M	molar
MES	2-[N-morpholino]ethanesulphonic acid
mg	milligram
mins	minutes
ml	millilitre
mM	millimolar
mm	millimetre
MMLV	Moloney murine leukaemia virus
MOPS	4-morpholinepropane sulphonic acid
μCi	microCurie
μg	microgram
μl	microlitre
μm	micron
μM	micromolar
N	amino
N	asparagine
N	Normal
NBT/BCIP	Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyphosphate p-toluidine salt, stable mix
ng	nanogram
NZY	NZ (hydrolysed casein)-yeast extract medium
P	proline
PBS	phosphate-buffered saline buffer
PCR	polymerase chain reaction
pfu	plaque-forming unit(s)
pmoles	picomoles
PKA	cAMP-dependant protein kinase
PKC	protein kinase C
PLM	phospholemman
PS	phosphatidyl serine
Q	glutamine
R	arginine
R	purine
RACE-PCR	rapid amplification of cDNA ends-PCR
rATP	adenosine 5'-triphosphate
RNA	ribonucleic acid

RNase	ribonuclease
RTPCR	reverse-transcription PCR
RT	reverse transcriptase
ryr	ryanodine receptor
S	serine
s	second(s)
Sarcosyl	N-lauryl sarcosine, Na^+ salt
SD	synthetic-dropout media
SD	Sprague-Dawley
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM	sodium- magnesium-chloride buffer
SSC	standard-saline-citrate buffer
SSPE	standard-saline-phosphate-EDTA buffer
T	threonine
T	thymidine
TBE	tris-borate-EDTA buffer
TBST	tris-buffered saline-Tween buffer
TdT	terminal deoxynucleotidyl transferase
TEMED	N, N, N', N'-tetramethylenediamine
Tm	transmembrane domain
Tm	melting temperature
Tris	tris[hydroxymethyl]aminomethane
Triton X-100	octylphenyl-nonaoxyethylene
Trp	tryptophan
Tween-20	polyoxyethylene-sorbitan monolaurate
U	unit(s)
UAS	upstream activating sequence
UTR	untranslated region
V	valine
V	Volt(s)
VDAC	voltage-dependant anion channel
v/v	volume per volume
W	tryptophan
W	Watt(s)
w/v	weight per volume
w/w	weight per weight
X	any nucleotide
X	any amino acid
X-Gal	5-bromo-4-chloro-3-inolyl-β-galactopyranoside
Y	pyrimidine
Y	tyrosine
YTH	yeast two-hybrid
3-AT	3-amino-1, 2, 4-triazole

Abstract

A cDNA clone, *p64H1,* was isolated from rat brain. This encoded a homologue of p64, a putative chloride channel protein. The work provided the first direct evidence of a p64-related gene in neural tissue, and evidence for the existence of a p64-related gene family. p64H1 was demonstrated to be an integral membrane protein of approximately 30 kDa. It contained a single transmembrane domain, a short luminal domain and a large cytoplasmic domain. The cytoplasmic domain was predicted to contain multiple consensus phosphorylation sites, and phosphorylation by Protein Kinase C was demonstrated *in vitro.* A polyclonal antiserum raised against a recombinant p64H1 fusion protein was used to investigate the cellular localisation of native and heterologously-expressed p64H1. The protein was expressed in a wide variety of tissues. Within rat brain, *p64H1* mRNA was found to be enriched in the cerebellum and hippocampus, and immunohistochemistry showed that the protein expression within the cerebellum was confined to the Purkinje cell layer. Recombinant p64H1 expressed in cultured cells was confirmed to be an endoplasmic reticulum membrane protein using indirect immunofluorescence. Finally, the interactions of p64H1 with other cellular proteins were investigated using both classical biochemical techniques and a yeast two-hybrid interaction assay. These varied approaches provided data which helped to characterise a previously undescribed protein, and may help in the elucidation of its precise cellular function.

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Chapter 1

Introduction

1.1 Ion channels

Ion channels are macromolecular pores which have been found in the membranes of every type of cell studied to date. Voltage-gated ion channels are responsible for the generation of electrical signals in cell membranes, and as such are central to the normal function of the nervous system. Other ion channels have similarly vital roles to play in normal cellular function. Channels are responsible for intraorganelle pH control, cell volume regulation, maintenance of membrane potential, release of hormones and transmitters and intracellular Ca^{2+}-signalling. The advent of recombinant DNA technology and the subsequent isolation of cDNA clones encoding a surprising variety of ion channels has led to an increased understanding of ion channel structure and function, and to the realisation that many ion channels have a common ancestral origin (Hille, 1992).

1.2 Structure-function relationships

1.2.1 Voltage-gated ion channels

A variety of plasma-membrane ion channels have been described, but the voltage-gated ion channels serve as an excellent model to describe the evolutionary conservation of ion channel design, and to illustrate the relationships between the pore-forming principle channel subunits and their accessory subunits. The voltage-gated sodium, potassium and calcium channels are responsible for the generation of conducted electrical signals in neurons and other excitable cells. Understanding the molecular bases for voltage-dependant activation, rapid inactivation and selective ion transport has been a major goal of current research on these proteins.

1.2.2 Voltage-gated sodium channels

The purification and subsequent cloning of sodium (Na^+) channels took advantage of the specific, high affinity binding of neurotoxins to the channel complex. The Na^+ channel from eel electroplax was purified using the binding of radiolabelled tetrodotoxin as a specific assay (Agnew *et al.*, 1978), and consisted of a single polypeptide of 280 kDa, similar in size to the rat brain sodium channel (Agnew *et al.*, 1980). The cloning of the eel electroplax Na^+ channel cDNA (Noda *et al.*, 1984) gave the first insight into the primary structure of a voltage-gated ion channel. Oligonucleotide-directed screening of various cDNA libraries, based upon amino acid sequence information obtained from purified electroplax protein, allowed the isolation of cDNAs encoding the entire polypeptide. The deduced amino acid sequence revealed a protein with four internally homologous domains, and multiple putative transmembrane domains (Noda *et al.*, 1984). The cDNAs encoding the electroplax Na^+ channel were used as probes to isolate cDNAs encoding three distinct, but highly similar, rat brain Na^+ channels (types I, II and III; Noda *et al.*, 1986 a; Kayano *et al.*, 1988). Subsequently, cDNAs encoding the type II rat brain Na^+ channel were used as probes to isolate cDNAs encoding Na^+ channel α-subunits from skeletal muscle and brain (Trimmer *et al.*, 1989; Kallen *et al.*, 1990; Rogart *et al.*, 1989). All these Na^+ channel α-subunits have a close structural relationship, and other Na^+ channels cloned to date, such as *Drosophila* channels (Salkoff *et al.*, 1987; Ramaswami *et al.*, 1989; Loughney *et al.*, 1989), all possess similar domain structures. The domain structure deduced from the primary sequence data of all the Na^+ channel α-subunit cDNAs cloned to date is illustrated in Figure 1.1, a. The *N*-

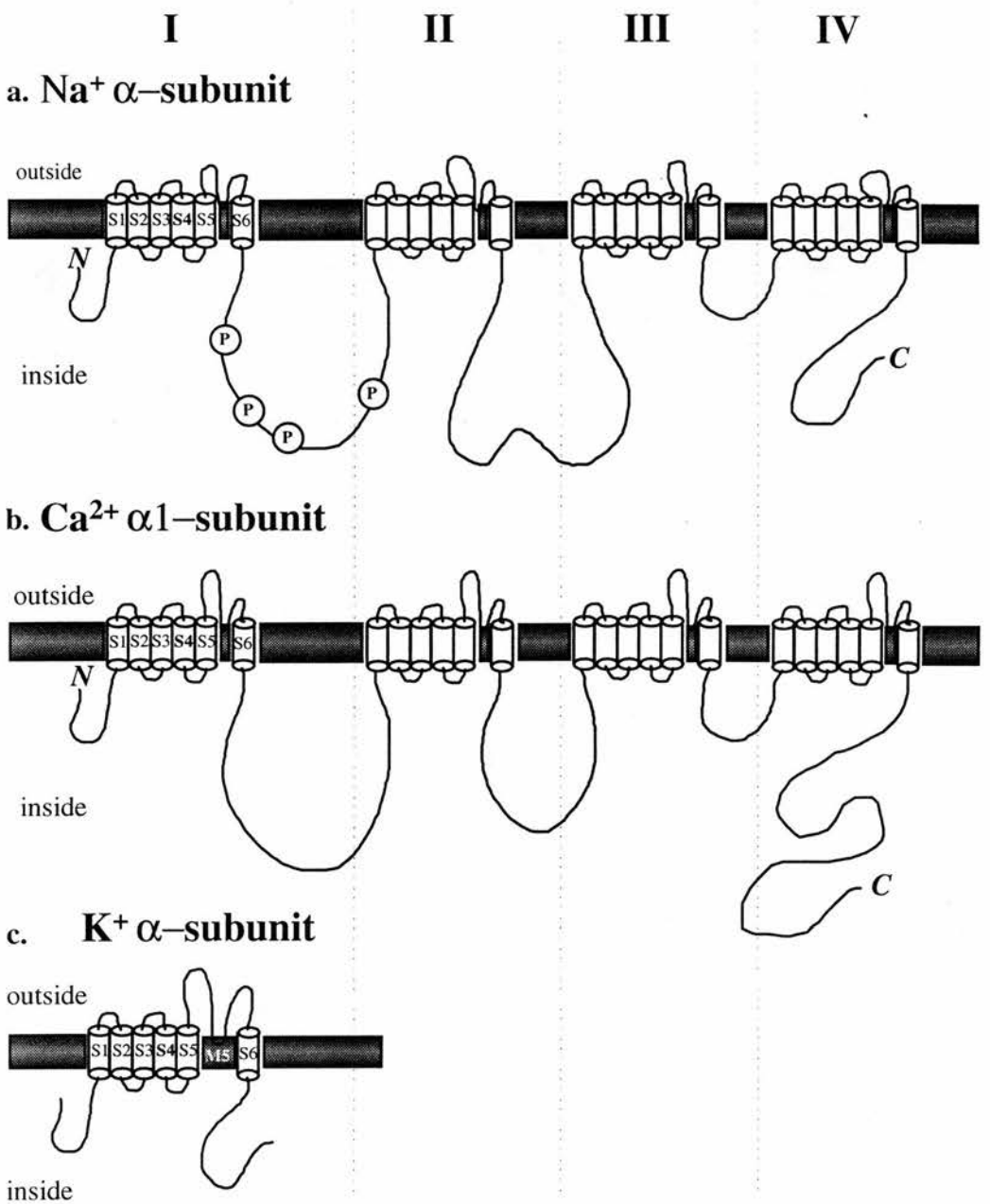


Figure 1.1 The structural similarities between voltage-gated Na^+, Ca^{2+} and K^+ channel α/α1 subunits. The repeated domains are indicated by the numerals I, II, III and IV. Consensus phosphorylation sites within the Na^+ channel are indicated by the symbol P (adapted from Hille, 1992).

and *C*-termini of the protein are both located cytoplasmically, with four homologous domains containing 6 transmembrane domains (designated S1 to S6) each predicted to form α-helices, and the stretch between S5 and S6 (known as H5 or P), separated by loop structures. The level of similarity between the cloned Na^+ channels is greatest between the transmembrane domains S1 to S6, whilst the level of conservation within the interconnecting loops is reduced.

1.2.3 The cloning of sodium channel accessory subunits

Studies involving the binding of labelled toxins to Na^+ channel subunits revealed that different toxins bound to different sites within the channel complex, with different perturbing effects on channel function and behaviour. The direct chemical identification of the rat brain β-subunit was achieved through the covalent binding of β-scorpion toxin (Jover *et al.*, 1988). Whereas measurement of the target size for inactivation of either tetrodotoxin or α-scorpion toxin binding to the Na^+ channel revealed a structure of 230 kDa to 266 kDa (Levinson and Ellory, 1973; Barhanin *et al.*, 1983), similar studies using β-scorpion toxin suggested a much smaller protein of 45 kDa to be involved (Angelides *et al.*, 1985). Purification of this β-subunit protein and amino acid sequence analysis led to the isolation of cDNA clones using a combination of PCR and cDNA library screening (Isom *et al.*, 1992). The deduced amino acid sequence of the β-subunit predicted a small (~ 23 kDa) protein with a short cytoplasmic domain, a single transmembrane domain and a large extracellular domain containing four consensus *N*-linked glycosylation sites. Early biochemical experiments suggested that the α-subunits of Na^+ channels were alone involved in

ion conductance and channel gating. Sodium channels purified from eel electroplax contained only α-subunits, but retained ion-conductance activity and a high affinity for tetrodotoxin (Lombet *et al.*, 1984; Correa *et al.*, 1990). The functions of the β-subunits were probed using purified proteins. The selective removal of the β-subunits from the detergent-solubilised αβ2 rat brain complex resulted in the complete loss of α-scorpion toxin binding and sodium transport activity (Messner *et al.*, 1986 a; 1986 b). Furthermore, the addition of tetrodotoxin quantitatively stabilised the αβ2 complex against the loss of the β-subunits and loss-of-function. Studies of the role of the β-subunits *in vivo* revealed that expression of the α-subunit from rat brain, heart and skeletal muscle alone in *Xenopus* oöcytes was sufficient to give rise to Na^+ currents, but that these currents had altered inactivation kinetics compared to the native, wild-type channels (Noda *et al.*, 1986 b; Suzuki *et al.*, 1988; Cribbs *et al.*, 1990; Patton *et al.*, 1994). Stable cell-lines of CHO cells expressing only rat brain α-subunits also gave rise to novel Na^+ currents in the absence of detectable endogenous β-subunits (West *et al.*, 1992), but when β-subunit cDNA was co-introduced, the kinetics of the measured channels changed significantly (Isom *et al.*, 1994). Thus, the role of the β-subunit in mammalian channel complexes may be to stabilise the complex in the plasma membrane, and also to regulate the voltage-dependence of the channel.

1.2.4 Structure and function of voltage-gated calcium channels

Like the Na^+ channel of eel electroplax, the calcium (Ca^{2+}) channels of the transverse-tubule membrane of skeletal muscle served as the primary biochemical

and molecular subject for study because of their abundance. The initial purification of these channels relied upon the high-affinity binding of dihydropyridine agonists (Curtis and Caterall, 1984; Borsotto *et al.*, 1985). These experiments demonstrated the presence of α-subunits of 175 kDa, together with accessory subunits of 143 kDa and 27 kDa (Leung *et al.*, 1987; Takahashi *et al.*, 1987). The α1-subunit of the skeletal muscle Ca^{2+} channel was cloned using oligonucleotide-directed cDNA library screening based on amino acid sequence data (Tanabe *et al.*, 1987). The deduced protein was 1873 amino acids in length, with a molecular weight of 212 kDa, larger than the estimate of 175 kDa for the α1-subunits of the purified protein. Further experiments demonstrated that the α1-subunits of the purified protein were truncated at their carboxy-terminal, resulting in a protein of approximately 190 kDa which migrated anomalously in SDS-PAGE (De Jongh, 1989), and that only a small fraction contained full-length protein. Furthermore, both forms have been detected in rat skeletal muscle cells, indicating that both forms may be present *in vivo* (Lai *et al.*, 1990).

The predicted membrane topology of the α1-subunit is similar to that suggested for the voltage-gated sodium channel, with four homologous domains, each containing six transmembrane domains, and linked by extracellular and intracellular loop regions (Figure 1.1, b). The 143 kDa subunit, designated α2, was cloned in a similar manner to the α1 subunit (Ellis *et al.*, 1988). The deduced protein was of 1106 amino acids, with a molecular weight of 125 kDa, multiple consensus glycosylation sites and multiple potential transmembrane domains (Ellis *et al.*, 1988). In addition to the α1 and α2 subunits described, skeletal muscle Ca^{2+}

channel β-subunit cDNAs were cloned, predicting a protein of 524 amino acids and a molecular weight of 58 kDa (Ruth *et al.*, 1989). The β-subunits are not glycosylated, suggesting that they reside on the intracellular side of the plasma membrane, and they contain multiple consensus sites for phosphorylation by protein kinase C (PKC) and cAMP-dependent protein kinase (PKA). The predicted amino acid sequence did not contain any potential transmembrane domains, suggesting that the β-subunit was a peripherally-associated membrane protein. The smallest subunit (δ) described biochemically was of 27 kDa. This was cloned and demonstrated to be encoded by the same mRNA transcript as the α1-subunit (Vandaele, 1987), arising after a truncation at alanine 934 of the α1δ precursor.

Several additional genes encoding the α1-subunits of Ca^{2+} channels have been identified by cDNA cloning, using the skeletal muscle cDNA as a probe. In general, the level of similarity between the subunit isoforms is greatest in the transmembrane domains, with reduced conservation in the large loops connecting domains I, II and III, and in the intracellular *N*- and *C*-terminal domains. In addition, most of the α1-subunit genes are alternatively-spliced, giving rise to additional subunits. Additional homologues of the skeletal-muscle β-subunit gene have been identified, and some of these are alternatively-spliced (Ruth *et al.*, 1989; Powers *et al.*, 1992; Pragnell *et al.*, 1991; Hullin *et al.*, 1992; Perez-Reyes *et al.*, 1992).

Like the α-subunits of Na^+ channels, the α1-subunits of some Ca^{2+} channels can serve as voltage-gated calcium channels when expressed alone (e. g. Perez-Reyes, 1989). The co-expression with other subunits, or reconstitution of native protein

complexes demonstrated that, like voltage-gated Na^+ channels, the auxiliary subunits had a substantial effect on the gating properties of the channel complex (e. g. Lacerda *et al.*, 1991; Welling *et al.*, 1993). Like the voltage-gated Na^+ channels, the Ca^{2+} channel α-subunit can function autonomously, but requires the accessory subunits for efficient expression and regulation. Similarly, Ca^{2+} channel α-subunits from tissue other than skeletal muscle can serve as voltage-gated channels when expressed alone in *Xenopus* oöcytes (e. g. the cardiac L-type channel, Mikami, 1989; smooth muscle and brain α-subunit isoforms, Itagaki *et al.*, 1992; Tomlinson *et al.*, 1993), but all are modulated by auxiliary subunit expression.

1.2.5 Voltage-gated potassium channel structure

The molecular structure of voltage-gated potassium (K^+) channels was first demonstrated by the cloning of the gene responsible for the *Shaker* mutation in *Drosophila* (Pongs, 1992; Jan and Jan, 1992). These channels have principle (α) subunits of 60 to 80 kDa which are homologous in structure to a single domain of the α- or α1-subunits of Na^+ and Ca^{2+} channels (Figure 1.1, c), containing 6 transmembrane domains (S1-S6). Potassium channel α-subunits form homo- and hetero-tetramers that are fully-functional as voltage-gated channels, but like voltage-gated Na^+ and Ca^{2+} channels, their regulation and modulation is affected by auxiliary β-type subunits. At least 18 genes exist encoding voltage-gated K^+ channel α-subunits in the mammalian nervous system alone: these belong to 6 families, corresponding to the six cloned *Drosophlia* potassium channel genes (Wei *et al.*, 1994; Chandy and Gutman, 1995; Trudeau *et al.*, 1995). In addition, 4

mammalian β-subunits have been reported (England *et al.*, 1995; McCormack *et al.*, 1995), which affect the inactivation, voltage-dependant activation, and the rate of de-activation of the α-subunit-encoded channel.

1.2.6 The evolutionary conservation of ion channel design

Sequence alignment of the voltage-gated Na^+, Ca^{2+} and K^+ channels clearly showed that they are evolutionary homologues. The conservation of the six-transmembrane (S1-S6) domain motif is striking between all members of the superfamilies. The functional significance of conserved domains has been well studied. The S1 domain is essential for the assembly of functional ion channels, and is highly hydrophobic (Babila *et al.*, 1994). There is much evidence to suggest that the S4 region functions as a voltage sensor (e. g. Bezanilla *et al.*, 1994; Gross *et al.*, 1993; Jan and Jan, 1992; Liman *et al.*, 1991). The consensus sequence of S4 domains (AILRVIRLVRVFRIFKLSRHSKGL; Montal, 1995) is striking in the conservation and regularity of a motif (RXX or KXX), in which X is usually a non-polar residue, and suggests that this motif may have descended from a single, ancestral voltage sensor motif (Montal, 1995). The loop connecting domains S5 and S6 (known as H5 or P) is the most conserved domain in all K^+ channels, and mutational analysis of sodium and calcium channels (Heinemann *et al.*, 1992; Yang *et al.*, 1993) suggest a similar, important role for this domain in ion selectivity in these channels. The identification of a K^+ channel in *E. coli* which contained this conserved domain (Milkman, 1994) suggests a common ancestor for the selectivity filter domain (Milkman, 1994). This conservation of ion channel design is retained in anion

channels, which usually contain multiple Tm domains and exist as members of superfamilies.

1.3 The ClC-chloride channel family

The ClC-family comprises the only large family of chloride (Cl^-) channels cloned to date. Members of this group display a high degree of evolutionary conservation, possessing some 12 Tm domains and functioning as multimers. In mammals, 9 different ClC channels have been identified, which may be assigned to one of three family branches based upon homology (Jentsch, 1996). The first branch, which is most closely related to the *Torpedo* ray channel ClC-0 (Jentsch *et al.*, 1990), includes ClC-1, present in skeletal muscle and whose inactivation causes myotonia (Steinmeyer *et al.*, 1994; Meyer-Kleine *et al.*, 1995), ClC-2 , a ubiquitously-expressed channel activated by cell-swelling (Grunder *et al.*, 1992), and two kidney-specific channels, ClC-Ka and ClC-Kb (known as ClC-K1 and ClC-K2 in rat, Kieferle *et al.*, 1994; Uchida *et al.*, 1994). The second branch includes ClC-3 and ClC-4 (Kawasaki *et al.*, 1994; Adachi *et al.*, 1994), both with widespread tissue distribution, and ClC-5, expressed mainly in the kidney, whose inactivation causes kidney stones (Lloyd *et al.*, 1996; Steinmeyer *et al.*, 1995). These three proteins are most closely related to a putative ClC-protein expressed in *S. cerevisiae* (scClC; Greene *et al.*, 1993). The third branch of the ClC family includes the *putative* channel proteins ClC-6 and ClC-7, which have not been expressed functionally as Cl^- channels (Brandt and Jentsch, 1995). All the members of the ClC-family share a similar membrane topology, comprising around 12 Tm domains (D1-D12; Jentsch

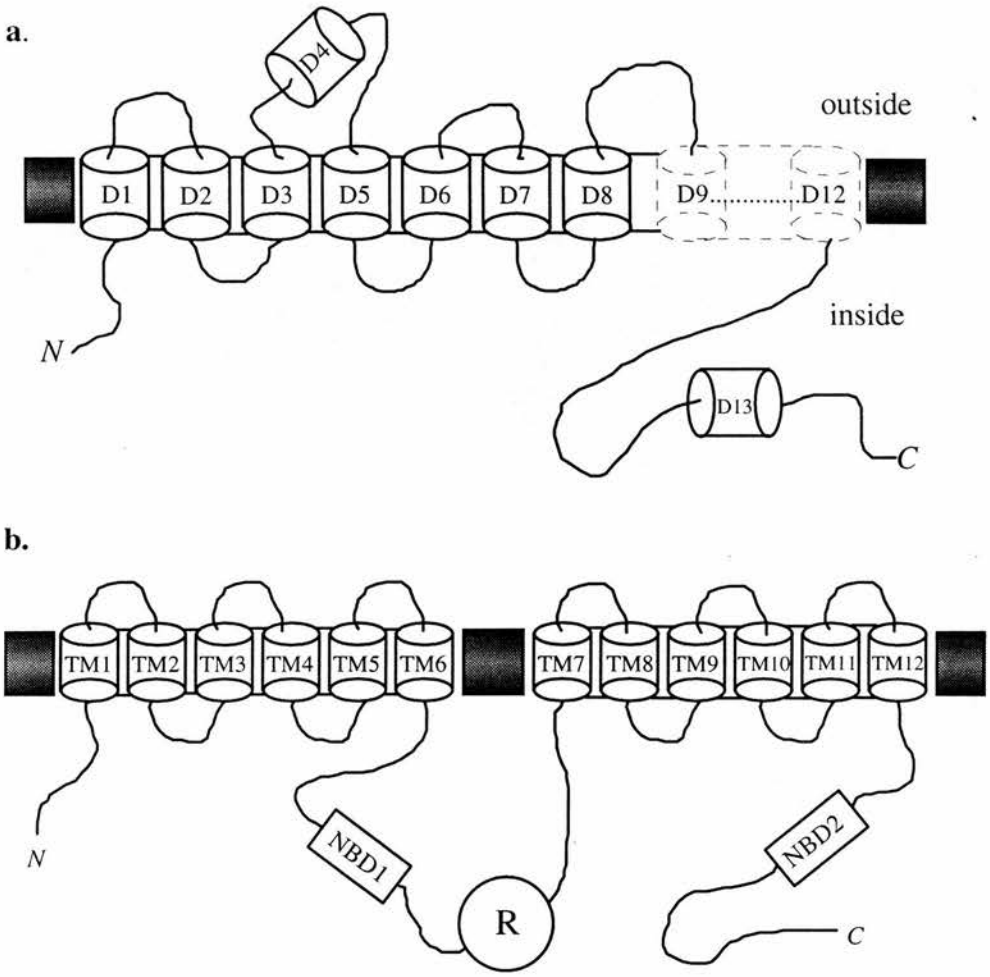


Figure 1.2 Predicted topologies of the ClC and CFTR channels.

a. Membrane topology model for the ClC channels (adapted from Jentsch, 1996). **b.** Membrane topology model for CFTR. The nucleotide-binding domains are labelled NBD1 and NBD2, and the regulatory domain is labelled R (adapted from Jentsch, 1996).

et al., 1993; Figure 1.2, a). The analysis of new ClC-genes suggested that domain D4 is not a conserved hydrophobic domain, and as such is unlikely to be a Tm domain (Brandt and Jentsch, 1995). Some controversy existed regarding the transmembrane property of the D2 domain (Middleton *et al.*, 1994), but it is generally accepted now to be a Tm domain. The exact membrane topology of the ClC-channels remains unknown, with the predictions regarding the regions D9 to D12 remaining obscure (Figure 1.2, a). The region D13 has, however, been demonstrated to be a cytoplasmic domain (Grunder *et al.*, 1992).

1.4 The Cystic Fibrosis Transmembrane Regulator (CFTR)

A concerted effort to identify the defective gene or genes responsible for cystic fibrosis (CF) led to the isolation of cDNAs encoding CFTR (Riorden *et al.*, 1989). Subsequently, it was demonstrated that CFTR was a nucleotide-regulated anion channel (e. g. Welsh *et al.*, 1992), and several hundred disease-causing mutations have since been identified at the CFTR locus (reviewed by Sheppard and Ostedgaard, 1996). CFTR comprises two homologous regions, containing 6 Tm domains and a single nucleotide-binding domain (Figure 1.2, b). These two regions are divided by a regulatory domain (R domain), which must be phosphorylated by cAMP-dependent protein kinase (PKA), to allow the channel to open. In addition to its ion channel function, an increasing number of reports suggest that the CFTR is a regulator of other ion channels. This hypothesis has been supported indirectly by the discovery that the sulfonylurea receptor (SUR), a member of the same superfamily (the traffic ATPases) as CFTR, regulates an inwardly-rectifying potassium channel

to give rise to ATP-sensitive potassium channels (Inangaki *et al.*, 1995). In addition, it has recently been discovered that CFTR regulates an outwardly-rectifying Cl^- channel expressed in many cell types (Egan *et al.*, 1992; Schwiebert *et al.*, 1995). In addition to defective chloride conductance, the airway epithelia of CF patients exhibit an increased Na^+ conductance and it has recently been demonstrated that CFTR leads to cAMP-stimulated inhibition of co-transfected epithelial Na^+ channels (Stutts *et al.*, 1994). The mechanisms involved in the interactions between CFTR and other ion channels or channel regulators remains unclear, but these data demonstrate the complexity of ion channel function and regulation.

1.5 Intracellular ion channels

Thus far, all the ion channel proteins discussed reside normally in the plasma membrane of cells. There is, however, increasing evidence for the existence of ion channels within intracellular membranes. These include ion channels in the endoplasmic and sarcoplasmic reticulum (e. g. Smith *et al.*, 1985; Ashley, 1989; Schmid *et al.*, 1988; Martin and Ashley, 1993), mitochondria (e. g. Sorgato *et al.*, 1987; Hayman *et al.*, 1993), secretory vesicles (e. g. Ashley *et al.*, 1994), and synaptic vesicles (Rahamimoff *et al.*, 1988). Information regarding the molecular identities of these ion channels, with a few notable exceptions discussed below, is largely absent.

1.5.1 Ion channels of the endoplasmic and sarcoplasmic reticulum

1.5.1.1 The Ryanodine receptor

Direct recordings of Ca^{2+} release channels were first made by Smith *et al.* in 1985 and 1986, who described adenine nucleotide-activated channels in skeletal muscle sarcoplasmic reticulum (SR). Further work demonstrated the Ca^{2+} release channel to be sensitive to the muscle-paralysing alkaloid ryanodine (Imagawa *et al.*, 1987), an observation crucial to the identification of the ryanodine receptor as the channel-forming protein. The large molecular weight of the ryanodine receptor monomer (450-550 kDa), purified by virtue of ryanodine binding, led to speculation that it comprised the so-called "foot" structure seen at the junction of terminal cisternae and transverse tubules (e. g. Block *et al.*, 1988). Purified ryanodine receptors were incorporated into planer-lipid bilayers where they formed Ca^{2+} release channels with properties similar to those seen in native SR vesicles (e. g. Hymel *et al.*, 1988). These experiments demonstrated that the "foot" structures, the calcium release channel and the ryanodine receptor contained the same protein. Three different ryanodine receptor genes have been identified in mammals, designated the skeletal, cardiac and brain isoforms or RyR-1, RyR-2 and RyR-3, respectively (McPherson and Campbell, 1993). In addition to these mammalian genes, a homologue isolated from *Drosophila*, the *dry* gene, encodes a 15 kb transcript showing a high degree of conservation with the mammalian genes (Hasan and Rosbash, 1992). The expression of each of the mammalian genes is not tissue-specific, but each protein product is enriched in certain tissues, as suggested by the nomenclature. Furthermore, the isolation of these cDNAs, and the tissue distributions of their

transcripts and protein products demonstrated that the ryanodine receptor is not confined to tissues requiring excitation-contraction coupling, but rather they comprise a super-family of intracellular Ca^{2+} release channels.

A number of secondary-structural features are shared by the RyR isoforms. A motif of approximately 100 amino acid residues is repeated four times throughout each RyR (Hakamata *et al.*, 1992; Nakai *et al.*, 1990; Otsu *et al.*, 1990; Takeshima *et al.*, 1989; Zorzato *et al.*, 1990), with each isoform predicted to have molecular weights of 550-565 kDa. None of the genes encode a signal sequence, suggesting that the *N*-terminus is cytoplasmic. The membrane topology and Tm domain arrangements of the receptor proteins remains somewhat unclear, but 12 potential Tm domains have been identified by Zorzato *et al.* in 1990, whereas only four Tm domains were suggested by Takeshima *et al.* in 1989. A more recent model proposed by Tunwell *et al.*, (1996) suggests a 6 Tm domain structure. All models agree that the *N*-termini and central regions of the proteins are hydrophilic, and as such reside cytoplasmically in the "foot" domain originally observed in sarcoplasmic reticulum preparations (Figure 1.3, a). The ryanodine receptor proteins have been demonstrated to interact with several proteins within their cellular environment, including calmodulin (Seiler *et al.*, 1984), FK506 binding protein (FKBP; Jayaraman *et al.*, 1992) and triadin (Brandt *et al.*, 1990).

1.5.1.2 Inositol 1, 4, 5-Trisphosphate (IP_3) Receptors

A second class of intracellular Ca^{2+} release channel, the IP_3 receptor (IP_3R), is responsible for the transduction of an increase in intracellular IP_3 concentration to a

calcium signal (Miyawaki *et al.*, 1990). Complementary DNA encoding three distinct receptor sub-types, IP_3R-I, IP_3R-II, and IP_3R-III have been cloned (Furuichi *et al.*, 1989, 1994), suggesting the existence of differential IP_3/Ca^{2+}-signalling. The IP_3R-I (of 2749 amino acid residues) contains a large *N*-terminal cytoplasmic domain comprising approximately 80% of the molecule, a membrane-spanning region consisting of 6 putative Tm domains clustered near the *C*-terminus, and a short *C*-terminal cytoplasmic tail (Figure 1.3, b; Furuichi *et al.*, 1989; Mignery *et al.*, 1990; Michikawa *et al.*, 1994). The central portion of the protein has been described as the "modulatory" domain (Furuichi and Mikoshiba, 1995), and contains multiple consensus phosphorylation sites. These features suggest that the IP_3R may be central to a variety of signal transduction pathways. The structural features of the IP_3R suggest that it may be classified in the superfamily of voltage-sensitive K^+, Na^+ and Ca^{2+} channels and some second messenger-gated channels (Jan and Jan, 1992). IP_3R-I is predominantly found within the Purkinje cells of the cerebellum, localised in both the dendrites and the soma (Furuichi *et al.*, 1993), principally in the smooth ER.

The IP_3R-II is composed of 2701 amino acid residues, and shares approximately 68% similarity with the IP_3R-I protein (Sudhof *et al.*, 1991). The IP_3R-III contains 2670 amino acid residues, and shares 62% similarity with IP_3R-I and 65% similarity with IP_3R-II. In addition, the ryanodine receptor family members have fragmentary sequence homology with the IP_3 receptors, with the homology concentrated in the ligand-binding domain and the Tm domains from M5 to the *C*-terminal of the proteins (reviewed by Furuichi and Mikoshiba, 1995). These data suggest that these areas are important for determining calcium-release channel function and identity.

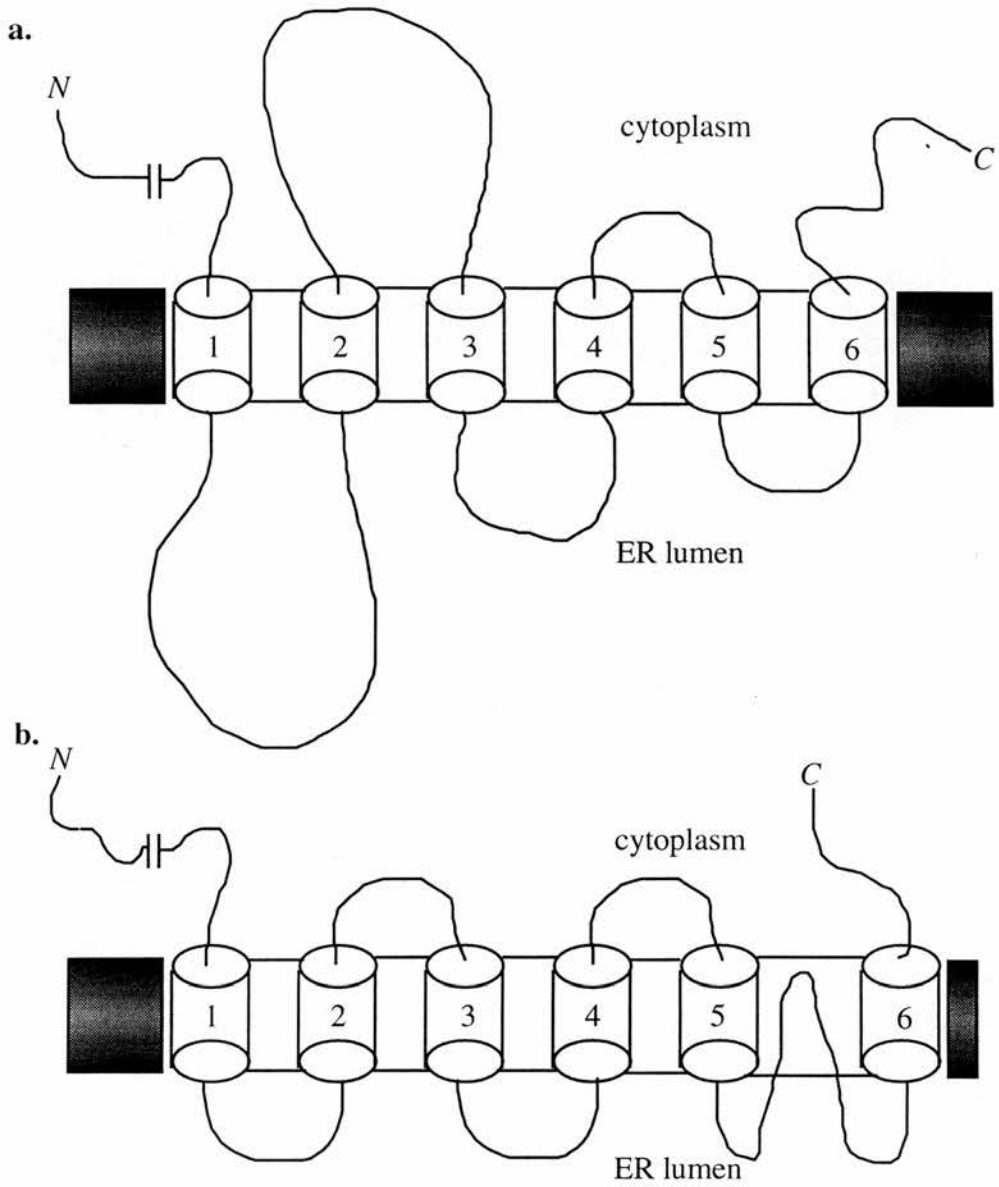


Figure 1.3 Structural similarities between the RyR and IP_3 receptors.

a. The topology model suggested by Tunwell *et al.,* for the cardiac RyR. The *N*-terminal region comprises ~90% of the entire protein, and resides in the "foot" domain (after Tunwell *et al.,* 1996). **b.** The model suggested for the transmembrane topology of the IP_3R suggested by Michikawa *et al.* The IP_3 binding site lies within residues 1-650, and the suggested pore-forming region lies in a pore-loop between Tm domains 5 and 6 (after Michikawa *et al.,* 1994).

1.5.2 The Voltage-Dependent Anion Channel

Voltage-gated anion channels (VDAC), or mitochondrial porins, are small (30 - 34 kDa), abundant proteins described in the outer mitochondrial membrane of all eukaryotic organisms studied to date (reviewed by Benz, 1994). Multiple VDAC genes have been described in sources as diverse as human (Blachly-Dyson *et al.*, 1993), potato (Heins *et al.*, 1994), mouse (Sampson *et al.*, 1996) and wheat (Elkeles *et al.*, 1995). Whereas the primary sequences are poorly conserved amongst these family members, the predicted secondary structure is remarkably preserved. In addition, the functional characteristics of each protein is highly conserved, demonstrated by the ability of cloned human VDAC to complement the phenotypic defect caused by deleting the VDAC gene in yeast (Blachly-Dyson *et al.*, 1989). All models of VDAC structure propose an amphipathic β-barrelled protein, with between 12 - 19 β-strands (e. g. Blachly-Dyson *et al.*, 1990; De Pinto *et al.*, 1991), similar to the structure described for the bacterial porins (Weiss *et al.*, 1991; Cowan *et al.*, 1992; Kreusch *et al.*, 1994) determined variously by protein crystallography and CD-spectra analysis. There is little primary sequence conservation between the porins and eukaryotic VDAC proteins, but functional characteristics seem to be remarkably preserved, raising the suggestion that there was a common ancestor for both groups of proteins (Berrier *et al.*, 1993). In this case, the porin channels would constitute a superfamily comprising porins from Gram-negative and certain Gram-positive eubacteria, Archaebacterial porins and channels found in organelles of eukaryotic cells (Berrier *et al.*, 1993). Within this super-family, the three-dimensional structure rather than the primary sequence would have been conserved

during evolution. This theory may be weakened by the obvious lack of data regarding the function of VDAC *in vivo* and the suggestion that the preparation method used to purify VDAC protein from tissue samples may have an effect upon the behaviour of the protein *in vitro* (Elkeles *et al.*, 1997), but serves to illustrate that protein function is by no means dictated by primary sequence.

1.6 A model ion channel?

All the channels discussed thus far contain pore-forming α-subunits possessing multiple transmembrane domains, often with associated, regulatory subunits which may or may not be integral membrane proteins. This is the pattern for most, but not all pore-forming ion channel subunits. The array of ion channels identified at the molecular level does contain channel proteins which do not conform to this pattern, some of which may serve to illustrate the technical difficulty in assigning ion channel function to membrane proteins expressed in heterologous systems.

1.6.1 Inwardly-rectifying potassium channels

Inwardly-rectifying K^+ channels allow potassium ions to enter a cell much more readily than K^+ permeation in the opposite direction, regardless of the potassium concentration in the external solution (Hagiwara *et al.*, 1976; Jan and Jan, 1997). The recent isolation of cDNA clones encoding at least 12 inwardly-rectifying K^+ channels (reviewed by Doupnik *et al.*, 1995) has revealed that these channel proteins are distinct from, but distantly-related to the voltage-gated K^+ channels. There is weak similarity between the hydrophobic domain of inwardly-rectifying K^+ channels

and the *C*-terminal half of the voltage-gated K^+ channels, suggesting that these regions are important for potassium permeation. These homologous hydrophobic domains form 2 α-helical Tm domains (M1 and M2) separated by a pore-loop domain (M5; Figure 1.4). A mutation in the H5 segment of the inwardly-rectifying channel GIRK2 abolishes potassium ion selectivity (Slesinger *et al.*, 1996), as does the analogous mutation in the *Shaker* H5 region (Heginbotham *et al.*, 1994). The hydrophilic *C*-terminal domain present in inwardly-rectifying, but not the voltage-gated K^+ channels, contains an acidic residue that is also important for inward-rectification and K^+ permeation (Taglialatela *et al.*, 1994; Yang *et al.*, 1995). This has raised the possibility of a pore-forming region in inwardly-rectifying channels extending further into the cytoplasm than is the case in voltage-gated K^+ channels.

1.6.2 The "Minimal" potassium channel, MinK

First cloned from rat kidney, (Takumi *et al.*, 1988) minK (or IsK) has been identified as a single-copy gene in rat, mouse, guinea pig and human (Murai *et al.*, 1989; Iwai *et al.*, 1990; Folander *et al.*, 1990; Lesage *et al.*, 1993; Zhang *et al.*, 1994). The deduced protein has 130 amino acids and a single transmembrane domain (Figure 1.5, a). Whereas the voltage-gated and the inwardly-rectifying K^+ channels share common molecular motifs in the K^+-pore region, the minK protein displays no relation to any known ion channel protein (Takumi *et al.*, 1988). For this reason, it was argued that the minK protein interacted with other, "traditional" K^+ channel subunits to form a functional ion channel. However, several groups of workers generated a compelling amount of data which appeared to support the model

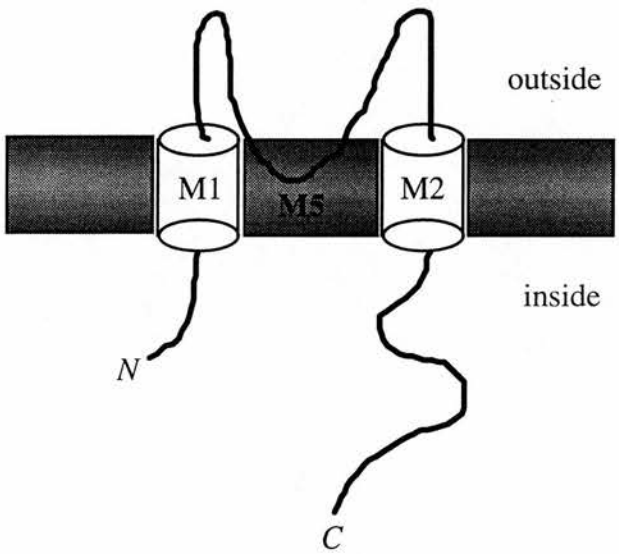


Figure 1.4 The predicted topology of inwardly-rectifying K^+ channels.

There is weak homology between the M1 and M2 domains of inwardly-rectifying and voltage-gated K^+ channels, and the M5 pore-loop domain appears to act as a selectivity filter in both sub-families.

(Adapted from Jan and Jan, 1997).

that minK alone could form K^+ channels in *Xenopus* oöcytes by dimerising (e. g. Wang and Goldstein, 1995), or by self-assembly into a channel complex of at least 14 subunits (Tzounopoulos *et al.*, 1995). Furthermore, point mutations in the putative Tm domain of minK resulted in alterations of conduction and pore selectivity (Goldstein and Miller, 1991; Wilson *et al.*, 1994), supporting the hypothesis that minK protein could form K^+ channels. MinK protein and mRNA have been detected in a variety of tissues, including brain, heart, kidney, colon and inner ear (reviewed by Busch and Suessbrich, 1997), suggesting an important, widespread function in ion conductance. The confusion surrounding minK was somewhat resolved by recent work which revealed that *in vivo* the minK protein itself is not a functional ion channel, as previously suggested by the observation that minK alone did not give rise to K^+ conductances in a variety of cell types (Lesage *et al.*, 1993; Barhanin *et al.*, 1996), but interacts with a pore-forming subunit of a K^+ channel, termed K_vQLT1 (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996). This channel protein had previously been described in patients affected by long-QT syndrome, an inherited cardiac arrhythmia (Wang *et al.*, 1996). The molecular mechanism underlying the association between minK and the K_vQLT1 channel remains unclear, but a physical interaction has been clearly demonstrated by co-immunoprecipitation assay (Barhanin *et al.*, 1996). The conflicting data regarding the function of minK alone as an ion channel illustrates the difficulty in ascribing ion channel function to a particular protein when it is expressed in a heterologous system.

1.6.3 Phospholemman

Phospholemman (PLM) is a small (~ 15 kDa) transmembrane protein isolated from canine cardiac sarcolemmal membranes before the subsequent cloning of its cDNA (Palmer *et al.*, 1991). The deduced amino acid sequence of phospholemman contains a single α-helical Tm domain, a larger cytoplasmic *C*-terminal domain and an extracellular *N*-terminal domain (Figure 1.5, b). PLM is the major plasmalemmal substrate for PKA- and PKC-mediated phosphorylation, and contains consensus phosphorylation sites for both these kinases in its cytoplasmic tail (Palmer *et al.*, 1991). PLM is widely-expressed, with a major hybridising mRNA species detected by Northern analysis in heart, skeletal muscle, stomach, oesophagus, aorta and liver tissues. Recently, a cDNA encoding a novel, PLM-like protein, Mat-8 was isolated from human breast tumours (Morrison *et al.*, 1995). Mat-8 is predicted to conform to a similar membrane topology to PLM, but has a different expression pattern as determined by Northern hybridisation analysis (Morrison *et al.*, 1995). Both PLM and Mat-8 induce hyperpolarization-activated Cl^- currents in transfected *Xenopus* oöcytes (Moorman *et al*, 1992; Morrison *et al.*, 1995), and point mutations introduced into the Tm domain region of PLM altered conductances (Moorman *et al.*, 1992). The underlying nature of these PLM- or Mat-8-induced Cl^- currents remains unclear. PLM and Mat-8 may be pore-forming subunits of Cl^- channels, or associate with other proteins within cells to form an active channel.

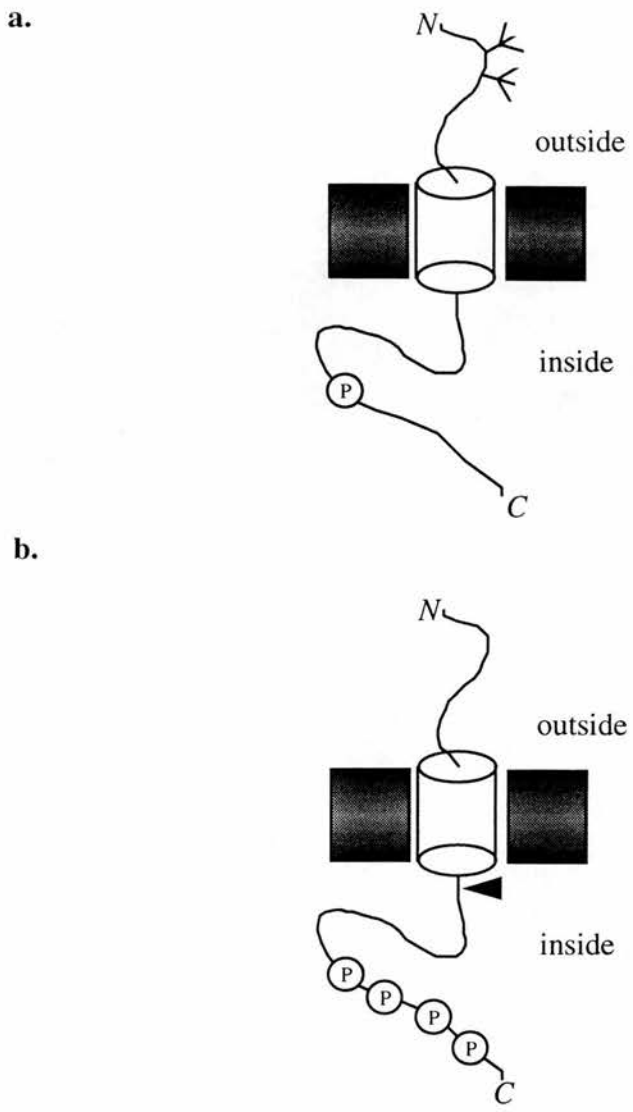


Figure 1.5 The predicted topologies of minK and PLM.

a. The predicted topology of minK. Consensus glycosylation sites are indicated by the "tree" symbol, and a potential PKC phosphorylation by the symbol P.

b. The predicted topology of phospholemman. The four consensus sites for PKC- and PKA-mediated phosphorylation are indicated by the symbol P. The arrow-head denotes a stop-transfer signal (RRCRCK) immediately adjacent to the Tm domain.

1.7 p64

The putative Cl^- channel p64 was originally described after partial purification and reconstitution of a 64 kDa (apparent molecular weight) protein from bovine kidney microsomes (Landry *et al.*, 1989). The purification strategy used was based upon ligand-affinity chromatography using synthetic, high-affinity ligands based upon indanyloxyacetic acid (IAA). The ligand IAA-94 was developed by screening inhibitors of Cl^- transport in proteoliposomes containing reconstituted protein from solubilised bovine kidney cortex membranes (Landry *et al.*, 1989). Potential-driven uptake of $^{36}Cl^-$ was observed when the solubilised proteins were incorporated into liposomes, which was inhibited by IAA-94 with an IC_{50} of ~ 1 μM. Subsequently, the ligand IAA-23 was used to affinity-purify proteins from kidney cortex, which were eluted using IAA-94 (Landry *et al.*, 1989). The eluate was found to be enriched for four protein species of 97, 64, 40, and 27 kDa, which were subsequently incorporated into proteoliposomes. Several distinct Cl^- channels were seen after fusion of these proteoliposomes with planar lipid bilayers, which were unaffected by the inhibitors IAA-94, stilbene isothiocyanates or potent anthranilic acid analogues. The Cl^- transport activity of the partially purified proteins was studied further after amino-acid sequence determination and the development of specific polyclonal antisera against one of the candidate species (Redhead *et al.*, 1992). Sequence-analysis of the *N*-terminal region of the 27- and 97 kDa protein species revealed their identities as glutathione-*S*-transferase and the α-subunit of the Na^+, K^+, -ATPase respectively, both abundant kidney proteins known to bind with high-affinity to IAA-23 analogues (Redhead *et al.*, 1992). Attempts to raise

polyclonal antisera against the remaining 2 candidates of 40- and 64 kDa were only partly successful, yielding a high-titre antibody against only the 64 kDa species (Redhead *et al.*, 1992). Attempts to reconstitute immuno-purified 64 kDa protein from kidney microsomes into planar-lipid bilayers never gave rise to Cl^- currents, but the antiserum was shown to immunodeplete Cl^- transport activity from bovine kidney microsomes. The conclusion from these data was that the 64 kDa protein species (p64) eluted from the ligand-affinity chromatography column was a component of a Cl^- channel. The anti-p64 polyclonal antiserum was used to immunolocalise p64 within CFPAC-1 cells, demonstrating p64 protein to be localised largely to the membranes of vesicular intracellular organelles (Redhead *et al.*, 1992).

Further to this work, a kidney cortex cDNA expression library was screened using anti-p64 antiserum, resulting in the isolation of a cDNA (H2B) which encoded p64 (Landry *et al.*, 1993). The cDNA clone obtained contained an insert of ~ 2.4 kb which encoded the entire ORF of p64. The predicted protein based upon the cDNA sequence of H2B had a molecular weight of 48 kDa, and hydrophathy predictions suggested the presence of 2 or 4 Tm domains. The deduced amino acid sequence of p64 showed no similarity to any other proteins in the SwissProt database. Northern hybridisation analysis demonstrated p64 mRNA to be expressed in bovine kidney medulla and cortex, liver, adrenal gland, brain, skeletal muscle and heart, as well as in a variety of cell-lines. The size discrepancy between the ORF-containing cDNA insert and the mRNA transcript was accounted for by an unusually large 3'-untranslated region. Complementary RNA derived from H2B cDNA was injected

into *Xenopus* oöcytes in order to assess the Cl^- transporting properties of p64 *in vivo*. A novel protein of the correct apparent molecular weight was detected in injected oöcytes, but no new plasma membrane Cl^- currents could be detected using the patch-clamp technique. Further study revealed that the expression of p64 was localised exclusively to intracellular membranes, suggesting that the absence of novel Cl^- currents was due to the internalisation of p64 protein. Recent work (Redhead *et al.*, 1997) has demonstrated that p64 is localised to dense-core vesicles which are part of the regulated secretion pathway. Furthermore, sequence signals within both the *N*- and *C*-terminal regions of p64 are required to direct this localisation. Recently, a cDNA encoding a novel homologue of p64 (NCC27; Valenzuela *et al.*, 1997) was cloned and functionally-expressed. The amino acid sequence deduced from this cDNA predicted a protein approximately half the size of p64, homologous to the *C*-terminal region, with two Tm domains. Sequence analysis predicted a nuclear-localisation signal within the protein, and indirect immunofluorescence experiments confirmed a nuclear membrane location for heterologously-expressed NCC27. Patch-clamp analysis of nuclear membranes from NCC27-transfected cells revealed the appearance of novel Cl^- channels not present in the nuclear membranes of control cells.

The likelyhood that p64 is not a Cl^- channel itself, but instead a Cl^- channel regulator or activator has never been addressed. The membrane topology of p64 has never been determined experimentally, but the suggested topology is unlike that observed for other Cl^- channel proteins. The work described in this thesis was directed towards the isolation and characterisation of a rat brain homologue of p64. The only

putative intracellular Cl^- channel protein to be cloned to date is p64. The existence of a p64-like family of proteins was predicted based upon the observation that every other ion channel protein identified is a member of a protein family. Furthermore, much of the data regarding bovine p64 is unreliable, and the experimental characterisation of either p64, or a closely-related protein may help elucidate the true function of these proteins.

As discussed previously, the primary sequence need not be the sole determinant of a protein's function. The secondary, tertiary and quaternary structural conformations of a particular protein may have as much to do with the ultimate function as the primary amino acid sequence. This is demonstrated by the VDAC family, whose members retain little sequence similarity, but great secondary structure homology. The determination of the membrane topology of p64 or of p64 homologues may provide clues as to the function of the protein *in vivo*. To date, the only protein ascribed Cl^- transport activity that has fewer than 2 Tm domains is PLM (Moorman *et al.*, 1992). The suggested membrane topology of p64 makes it seem unlikely that p64 alone is a Cl^- channel, but further experimental work is required to fully resolve this problem.

Chapter 2

Materials and Methods

2.1 *Eschericia coli* strains used

XL1-Blue MRF': *Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac[F' proAB, lacI^{q}ZΔM15, Tn10]*

JM109: *endA1, recA1, gyrA96, thi, hsdR17, (r_k^-, m_k^+), relA1, supE44, Δ(lac-proAB), [F', tra D36, proAB, lacI^{q}ZΔM15]*

HB101: *thi-1, hsdS20 (r_b^-, m_B^-), supE44, recA13, ara-14, leuB6, proA2, lacY1, rps L20, (str^r), xyl-5, mtl-1*

C600 *hfl:* *thi-1, thr-1, leuB6, lacY1, tonA21, supE44, hflA150, [chr::Tn10]*

SOLR: *e14$^-$(mcrA), Δ(mcrCB-hsdSMR-mrr) 171, sbcC, recB, recJ, umuC::Tn5, uvrC, lac, gyrA96, relA1, thi-1, endA1, λ^R, [F' proAB, lacI^{q}ZΔM15] su$^-$*

2.2 *Saccharomyces cerevisiae* strains used

CG1945: **MATa**: *ura3-52, his3-200, lys2-801, ada2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, cyh^{r}2*, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3::GAL4$_{17\text{-}mers(x3)}$-CyC1$_{TATA}$-lacZ

2.3 Materials and suppliers

2.3.1 Chemicals

All chemicals were supplied by the Sigma Chemical Company except where otherwise stated, and were of the highest grade available.

2.3.2 Molecular Biology Reagents

Amersham, Little Chalfont, Bucks, UK.

All radiolabelled nucleotides and amino acids and hybridisation membranes. Membranes for Western blotting and ECL detection kit. Sequenase™ II DNA Sequencing kit.

Boehringer Mannheim, Lewes, Sussex, UK.

Calf Intestinal Alkaline Phosphatase, Expand™ Long PCR polymerase mix.

Clontech, La Jolla, CA

Rat whole brain 5'-STRETCH cDNA library constructed in λgt 10, Rat whole brain yeast two-hybrid library and associated yeast strains, Rat Multiple Tissue Northern blot.

Flowgen, Staffordshire, UK.

Sequagel™ polyacrylamide reagent system. SeaKem™ LE agarose.

GIBCO BRL, Paisley, UK.

All reagents and media for mammalian cell culture, Superscript II™ RNase H$^-$ MMLV-reverse transcriptase, terminal deoxynucleotidyl transferase (TdT)., NBT/BCIP stable mix for alkaline-phosphatase detection, 1 kb DNA ladder.

New England Biolabs, Beverly, MA

T4 RNA Ligase.

Pharmacia Biotech, St. Albans, UK.

Random hexadeoxynucleotides, dNTPs.

Promega Corporation UK, Southampton, UK.

All restriction endonucleases, T4 DNA ligase, *Taq* DNA polymerase, TnT™ coupled transcription/translation kit. Plasmid vectors pCI and pGEM-T.

QIAGEN Ltd, Dorking, Surrey, UK.

Qiagen 500 plasmid purification kits, miniprep spin columns, QIAEX II DNA extraction kit.

R & D Systems, Cambridge, UK.

Plasmid vector pTAg.

Stratagene, La Jolla, CA

ExAssist™ Helper phage, plasmid vector pBluescript II KS (+/-), rat brain protein kinase C, Poly (A)-Quik™ mRNA purification columns, *E. coli* strains XL1-Blue and SOLR.

2.3.3 Culture media

All media components were from the Bacto™ range, purchased from Difco Laboratories, Surrey, UK. Media were prepared according to standard recipes (Sambrook *et al.*, 1989; pp A1-A5), except where otherwise indicated.

2.3.4 Deoxyoligonucleotide synthesis

All DNA synthesis was performed by OSWEL Ltd., Southampton, UK.

2.3.5 Antibodies

Biosource International, Inc., Camarillo, CA.

Goat anti-rabbit IgG (H + L) human adsorbed, Rhodamine-B conjugate.

Clontech, La Jolla, CA.

Mouse monoclonal anti-GAL 4 DNA BD, horseradish peroxidase conjugate.

Pierce & Warriner, Chester, UK.

Goat anti-mouse IgG (H + L), horseradish peroxidase conjugate, goat anti-rabbit IgG (H + L), horseradish peroxidase conjugate.

Sigma Chemical Co., Dorset, UK.

Donkey anti-rabbit IgG (H + L), alkaline phosphatase conjugate.

2.4 Standard recombinant DNA protocols

Several of the protocols used are described elsewhere (Sambrook *et al.*, 1989). These included phenol extraction of nucleic acids (pp E3-E4), nucleic acid precipitation (pp E10-E15), agarose gel electrophoresis (pp 6.9-6.12), restriction endonuclease digestion (pp 5.28-5.32) and autoradiography (pp E21-E27). DNA was recovered from agarose gels using a QIAEX II kit according to the manufacturer's instructions. Competent *E. coli* (XL1-Blue) were prepared and transformed according to the protocol described in the Promega Protocols and Applications Guide (pp 45-46). Occasionally, a commercially prepared stock of competent JM109 cells (Promega) was also used. The establishment and maintenance of bacterial and yeast cultures followed standard microbiological procedures described elsewhere (e. g. Sambrook *et al.*, 1989; p 1.21).

2.5 Cloning in plasmid vectors

Directional cloning was used throughout this study wherever possible, but on occasion cloning was performed where only a single restriction enzyme was used to prepare the vector and insert DNA. T/A-type cloning was also commonly used. This took advantage of the single base-overhangs added to the 3'-ends of PCR products by some thermostable polymerases in a template-independent manner (Clark, 1988). Several linear vectors were available which simplify the cloning procedure of these PCR products by possessing single T-overhangs (e. g. pGEM-T, Promega; pTAg, R & D Systems), meaning that such vectors could be ligated to the DNA insert without restriction digestion or other modification.

2.5.1 Preparation of the DNA fragment for "sticky-end" ligation

The DNA to be cloned was purified from an agarose gel using the QIAEX II silica resin method. After purification, the DNA was digested with the appropriate restriction endonuclease in order to generate compatible ends to the vector. Where two restriction enzymes were used, the digestion reactions were performed sequentially, and followed by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. Recovery of the digested DNA was confirmed by agarose gel electrophoresis.

2.5.2 Preparation of vector DNA for cloning

The strategies for preparing insert DNA for cloning were applied to vector preparation. Where a single restriction enzyme site was used, the digested vector

was treated with calf intestinal alkaline phosphatase (CIAP) to remove phosphate groups from 5'-termini. This treatment prevented the digested vector from re-ligating to itself.

2.6 Dephosphorylation of 5'-ends

The following components were added to the digested vector DNA: 10 µl CIAP 10 X buffer (320 mM ammonium sulphate, 1 mM $MnCl_2$, 0.1 mM $ZnCl_2$, pH 7.0) and 1 µl CIAP (1U/µl). Deionized water was added to a final volume of 100 µl. The samples were incubated at 37°C for 30 mins before phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. Samples were resuspended in deionized water to give a final DNA concentration of 50 ng/µl.

2.7 Ligation of plasmid vector and insert DNA

Concentrations of vector and insert DNA were estimated by electrophoresis and comparison with DNA molecular weight markers of known concentration. Various vector:insert ratios were tested to find the optimum ratio for each particular vector and insert. In most cases, a molar mass ratio of 1:1 or 1:3 was found to work well. The molar mass ratio for DNA molecules was estimated using the following formula; [ng vector x insert size (kb)/ vector size (kb)] x molar ratio of insert = ng of vector required. Individual ligation reactions were set up using the appropriate vector:insert ratios in a final volume of 10 µl. The reactions were performed according the T4 DNA ligase manufacturer's protocol (Promega). The ligation reactions were incubated at 16°C overnight, and terminated by incubation at 65°C for 10 mins.

2.7.1 T/A-type ligation reactions

Two T/A-type cloning vectors were used: pGEM-T (Promega) and pTAg (R&D Systems), taking advantage of template-independent 3'-tailing of PCR products with adenosine residues by some thermostable polymerases (Clark *et al.*, 1988). These vectors were comparable and were treated in the same way. The PCR product to be cloned was isolated from an agarose gel using the QIAEX II silica gel system, and the concentration estimated from an agarose gel as before. The ligation reaction was identical to that for "sticky-end" ligation, except that the incubation temperature for ligation was reduced to 4°C.

2.8 Analysis of transformants

2.8.1 Small-scale plasmid preparation: "minipreps"

The most commonly used technique to analyse recombinants was to purify plasmid DNA from individual clones and identify the insert in the recombinant plasmid by restriction digestion and agarose gel analysis. Three different "miniprep" protocols were used in this study, two of which are described elsewhere; the Boiling method (Sambrook *et al.*, 1989; pp 1.29-1.30) and the Alkaline Lysis method (Sambrook *et al.*; 1989; pp 1.25-1.28). For speed and convenience, a commercial kit (QIAGEN miniprep spin columns) was used on occasion to purify plasmid DNA from similar sized cultures, and the manufacturer's instructions were followed precisely. Ideally, the restriction enzyme(s) used to prepare the vector and insert DNA for ligation were used to digest the purified plasmid DNA. Where this was not possible (e. g. where the sequence of the insert was not known, or a T/ A type cloning vector was used),

digestion was performed using a restriction enzyme known to cut at a unique site within the vector.

2.8.2 Analysis of transformants by PCR

The analysis of transformants using PCR offered a rapid and convenient technique for the identification of recombinant clones (Gussow *et al.*, 1989). Not only was it possible to identify the clones containing the insert DNA, it was also possible on occasion to determine the orientation of the insert within the vector. This technique was used several times in this study, as follows: the components of the PCR reaction were assembled in a 0.5 ml tube and a small part of an individual clone was picked from the agar plate into the mixture using a sterile pipette tip. PCR was performed using appropriate thermal cycling conditions and the products were analyzed by agarose gel electrophoresis. By using one insert-specific primer, and one vector specific primer (flanking the multiple cloning site), the orientation of the insert in the vector could be determined.

2.8.3 Large scale preparation of plasmid DNA

The large-scale preparations followed the Alkaline Lysis protocol (Sambrook *et al.*, 1989; pp 1.25-1.28). QIAGEN 500 tips were also used according to the manufacturer's instructions.

2.9 Screening cDNA libraries constructed in λ phage vectors

2.9.1 Preparation of host cells

λZAP II: XL1-Blue MRF'

λgt 10: C600 *hfl*

The required strain was streaked on an LB-agar plate supplemented with the appropriate antibiotic (both these strains were tetracycline resistant) and incubated at 37°C overnight. Fifty millilitres of LB containing 0.2% (v/v) maltose and 10 mM $MgSO_4$ were innoculated with a single colony from the plate and incubated at 30°C overnight. The cells were collected by centrifugation at 2000 rpm for 10 mins and resuspended in approximately 15 ml of 10 mM $MgSO_4$ before further dilution until the OD_{600} reached 0.5.

2.9.2 Titering procedure

A quantity of host cells were prepared as described above. Serial dilutions were made of the stock library in SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 120 mM $MgSO_4$, 0.01% (v/v) gelatin) and 1 µl of diluted phage was added to 200 µl (for 90 mm plates) or 600 µl (for 150 mm plates) of host cells. The phage and bacteria were incubated together for 15 mins at 37°C to allow the phage to attach to the cells. Three millilitres (for 90 mm plates) or 7-8 ml (for 150 mm plates) of molten Top NZY-agarose (held at 54°C) were added to the phage/bacteria suspension and poured over dry NZY agar plates. The plates were incubated at 37°C until plaques began to appear in the bacterial lawn. The number of plaques on each plate was counted and the plaque-forming units per millilitre (pfu/ml) determined.

2.9.3 Preparation of radiolabelled DNA probes

With the exception of the oligonucleotide probe used in mRNA *in situ* hybridisation, all the probes used in the course of this study were derived from denatured, double-stranded DNA fragments labelled using the random-primed method (Feinberg and Vogelstein, 1983), in which a mixture of random hexamers was used to prime DNA synthesis *in vitro* from a linear DNA template. Template DNA (25 ng) in a total volume of 34 µl was denatured by boiling and rapid chilling on ice. The following components were added: 10 µl of oligo labelling buffer (250 mM Tris-HCl, pH 8.0, 250 mM $MgCl_2$, 10 mM DTT, 1 mM HEPES, 26 A_{260} units of random hexadeoxynucleotides), 2 µl BSA (10 mg/ml), 2 µl Klenow enzyme (1U/µl), 2 µl dNTPs (500 µM each of dATP/dGTP/dTTP, no dCTP present) and 5 µl [α^{32}P]-dCTP (1000 Ci/ml). The labelling mixture was incubated at room temperature for 2-3 h, before termination by the addition of 146 µl of DEPC-treated deionized water.

2.9.4 Removal of unincorporated label from labelling reactions

Unincorporated labelled dNTP's were removed from the labelling reaction before the probe was added to the hybridisation solution by passage over Sephadex G-50 spin columns. The columns were made as described (Sambrook *et al.*, 1989; p E38).

2.9.5 cDNA library screening protocol

The library titre was determined as described above, and 150 mm NZY-agar plates were prepared to 50 000 pfu/plate using 600 µl of host cells and 7-8 ml of top agarose per plate. The plates were incubated at 37°C for 8-10 h until the plaques

began to touch each other. The plates were removed from the incubator and chilled at 4°C for at least 2 h before a disc of Nylon membrane (Hybond-N, Amersham) was laid over the plate and the orientation marked by piercing through the membrane into the agar with a syringe needle. The membrane was left in contact with the plaques for 1 minute. The membrane was transferred, plaque-side-up, to a dish containing Denaturing solution (1.5 M NaCl, 0.5 M NaOH) and incubated for 7 mins without agitation. The membrane was transferred to another dish containing Neutralisation buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) and incubated for 6 mins. The membrane was transferred to a dish containing 2 X SSC (20 X stock: 3 M NaCl, 0.3 M Na Citrate, pH 7.5) and rinsed briefly. While still damp, the DNA was cross-linked to the membrane by exposure to UV light using a Stratalinker (Stratagene). The membranes were pre-hybridised, hybridised, probed and washed as described in Chapter 2.19.

2.9.6 Picking positive plaques

After autoradiography, any positive signals (seen as spots of variable intensity) on the film were aligned with the nylon membranes. The positions of the orientation holes were marked on the film. The marked film was aligned with the relevant plate. Using a Gilson P1000 disposable pipette tip, the top and bottom agarose was pierced to remove a "core". This was placed in a 1.5 ml tube containing 500 µl of SM buffer and 20 µl of chloroform and incubated at 4°C for at least 4 h to allow the phage particles to diffuse out of the agar core. The titre of each isolated core was determined, and phage plated out again at greatly reduced plaque density (250-500

pfu/150 mm plate). The plates were screened as before, and positive plaques isolated. Final agar cores were picked from well isolated plaques.

2.9.7 Characterisation of phage vector inserts

2.9.7.1 λZAP II

When the λZAP II cDNA library was used, phagemid DNA was rescued by *in vivo* excision using helper phage, adhering to the manufacturer's protocol (Stratagene Instruction Manual).

2.9.7.2 Characterising phage vector inserts from λgt 10

Inserts from isolated phage plaques were amplified using PCR and two vector-specific oligonucleotide primers. An aliquot (1 µl) of SM buffer containing the plaque of interest was frozen in liquid nitrogen then quickly thawed at 37°C to lyse the phage particles. This lysate was added to an LDPCR mixture, and the PCR products analyzed by agarose gel electrophoresis. Products were then cloned into the vector pTAg.

2.10 DNA Sequencing

All DNA sequencing reactions performed in this study used the Sequenase II kit (Amersham). This kit is designed primarily for the sequencing of single stranded templates; however, a protocol is included to denature double-stranded templates. This was found to be time-consuming, so a modified protocol was followed. The addition of 10% (v/v) DMSO (final concentration) to the denatured template DNA

prevented the re-annealing of the plasmid (Winship, 1989). Performing the termination reactions in the wells of a micro-titre plate further simplified the procedure (Sambrook *et al.*, 1989; pp 13.65-13.70). The following were assembled in a screw-cap tube: 4.5 µg of plasmid DNA, 10 pmoles of oligonucleotide primer and deionized water to a final volume of 8.25 µl, boiled for 3 mins and chilled rapidly on ice for 5 mins. The following were added directly, at room temperature: 1.25 µl of DMSO, 2.5 µl of *Sequenase* reaction buffer. Two-point-five microlitres of each termination mixture was added to individual, labelled wells in a microtitre plate and pre-warmed by floating the plate in a 37°C water bath. The following additions were made to the denatured DNA/primer mix: 1 µl DTT (0.1M), 2 µl *Sequenase* labelling mix (diluted 1:5 in deionized water), 2 µl *Sequenase* enzyme (diluted 1:7 in Dilution buffer) and 0.5 µl [α^{35}S]-dATP. The extension reaction mixture was incubated at room temperature for 5 mins. Three-point-five microlitres of this mix were added to each of the wells containing the termination mixes, mixed briefly by pipetting, and returned to the 37°C water bath for a further 5 mins. After this time, 4 µl of stop solution were added to terminate the reaction.

2.11 Denaturing polyacrylamide gel electrophoresis

Every denaturing polyacrylamide gel poured in this study used a commercially prepared gel component system: Sequagel (Flowgen). The components of this system were mixed in order to give a uniform 6% (w/v) polyacrylamide gel. Gels were cast in a BioRad SequGen apparatus, using 0.4 mm uniform spacers and a sharks-tooth comb. Samples to be electrophoresed were heat denatured before

loading by placing the micro-titre plate on a heated block at 90°C for 3 mins, then chilling quickly on ice. The gels were pre-run in 1.X TBE (8 mM Tris-HCl, 9 mM borate, 0.25 mM EDTA, pH 8.4) at a constant power of 75 W for 30 mins to equilibrate and pre-warm. Immediately before loading, the wells of the gel were flushed with 1 X TBE to remove excess urea, and 2.5 µl of each termination reaction were loaded in each well. Gels were run at 75 W constant power to maintain an even gel temperature throughout the run. When the bromophenol blue dye front reached the bottom of the gel, the run was stopped and the gel was dismantled before fixation in 10% (v/v) methanol, 10% (v/v) acetic acid for 5 mins. The gels were then dried under vacuum with a paper backing before autoradiography.

2.12 Protein translation *in vitro*

2.12.1 Coupled transcription and translation

The following were assembled in a sterile 1.5 ml tube:

TnT lysate	12.5 µl
TnT reaction buffer	0.5 µl
Amino acid mixture minus methionine	0.5 µl
RNasin ribonuclease inhibitor (40 U/µl)	0.5 µl
TnT T7 RNA polymerase	0.5 µl
^{35}S methionine (10 mCi/ml)	2.0 µl
RNase-free water	5.5 µl
plasmid DNA (1 µg/µl)	1 µl
(Optional) Canine pancreatic	

microsomal membranes	2.5 µl

Nuclease-free deionized water was added to make the final volume up to 50 µl. The mixture was incubated at 30°C for 45 mins. Aliquots (5 µl) were taken and analyzed by SDS-PAGE and autoradiography.

2.12.2 Localisation of p64H1 to microsomal membrane vesicles

In order to determine whether or not the translation products were associated with the canine pancreatic microsomal vesicles, aliquots of the translation mix (20 µl) were subjected to centrifugation at 100 000 x *g* for 5 mins at 4°C to pellet the microsomes. After centrifugation, the supernatent was discarded, and the pellet resuspended in SDS loading buffer and analyzed by SDS-PAGE and autoradiography.

2.12.3 Treatment of microsomal vesicles at high pH to disrupt vesicle contents and dissociate peripheral membrane proteins

Aliquots of the translation mix (20 µl) were incubated with 10 volumes of disruption buffer (0.1 M Na_2CO_4, pH 11.5) on ice for 30 mins before pelleting by centrifugation at 100 000 x *g* for 15 mins at 4°C. The pellets were rinsed with 10 volumes of PBS before resuspension in SDS loading buffer and analysis by SDS-PAGE and autoradiography.

2.12.4 Proteinase K protection assay

Aliquots of the translation mix (20 µl) were incubated with proteinase K (10 µg/ml) on ice for 30 mins, either in the presence or absence of 0.1% (v/v) Triton X-100.

The proteolysis was terminated by the addition of AEBSF (to 0.1 mM final concentration) and continued incubation on ice for a further 5 mins. Aliquots were analysed by Tris-tricine SDS-PAGE and autoradiography.

2.12.5 *In vitro* phosphorylation by protein kinase C (PKC)

In order to study the phosphorylation of p64H1 *in vitro*, the TnT buffer had to be replaced with a buffer suitable for PKC activity. This was achieved effectively by pelleting the microsomal membranes by centrifugation at 100 000 x *g* for 30 mins at 4°C, and discarding the supernatent. The pellet was resuspended in the following mixture:

sterile, deionized water	4.5 µl
10 X reaction buffer (400 mM MES, pH 6.0, 100 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM EGTA)	3.0 µl
rATP (1 mM)	4.0 µl
phospholipid solution (diolein and PS, Stratagene)	3.0 µl
PKC (Stratagene)	0.5 µl

The mixture was incubated at 30°C for 10 mins. The reaction was terminated by the addition of SDS-loading buffer before aliquots were analyzed by SDS-PAGE and Western blotting.

2.13 SDS-polyacrylamide gel electrophoresis of proteins

The electrophoresis of proteins followed the method of Laemmli (1970), with minor modifications.

2.13.1 Pouring of SDS-polyacrylamide gels

SDS-polyacrylamide gels were poured in a Hoeffer SE 600 apparatus, assembled according to the manufacturer's instructions. The solutions for preparing the SDS-polyacrylamide gels were assembled in the order shown;

	percentage acrylamide required		
component	10%	*12%*	*15%*
	ml	ml	ml
H_2O	11.9	9.9	6.9
40% acrylamide mix (BioRad)	10.0	12.0	15.0
1.5 M Tris-HCl, pH 8.8	7.5	7.5	7.5
10% (w/v) SDS	0.3	0.3	0.3
10% (w/v) ammonium persulphate	0.3	0.3	0.3
TEMED	0.012	0.012	0.012

The resolving gel was poured between the glass plates of the gel apparatus and overlaid with butanol saturated with water. After the resolving gel had polymerised, the overlay was removed and the stacking gel was poured over. Before the stacking

gel polymerised, the comb was inserted to allow the wells to form. The stacking gel was prepared by assembling the following in the order shown;

component	*ml*
H_2O	6.8
40% acrylamide mix	1.7
1.0 M Tris-HCl, pH 6.8	1.25
10% (w/v) SDS	0.1
10% (w/v) ammonium persulphate	0.1
TEMED	0.01

The samples were denatured by heating at 100°C for 5 mins in 1 X SDS gel sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol). Once the stacking gel had polymerised, the comb was carefully removed and the wells flushed first with deionized water and then with running buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.1% (w/v) SDS) to remove unpolymerised acrylamide. After heat denaturation, the samples were loaded and voltage applied to the gel at 8 V/cm until the dye front had moved into the stacking gel, and then 15 V/cm until the dye front reached the bottom of the resolving gel.

2.14 Tris-tricine SDS-PAGE

Gels (10%) were poured in the same apparatus as standard SDS-polyacrylamide gels, prepared according to the method of Shägger and von Jagow (1987).

2.15 Staining SDS-polyacrylamide gels with Coomassie Brilliant Blue

SDS-polyacrylamide gels were immersed in Coomassie dye (0.25% (w/v) Coomassie Brilliant Blue R250 in 90% (v/v) methanol/10% (v/v) glacial acetic acid) for 4 hours at room temperature. The gels were then destained by immersing in 90% (v/v) methanol/10% (v/v) glacial acetic acid for 24 hours, changing the destaining solution every few hours. After staining, gels were dried using a heated bed vacuum gel drier (BioRad).

2.16 Transfer of proteins from gels to solid supports: immunological detection of immobilised proteins (Western blotting)

The transfer of proteins from a gel to a membrane was achieved by electrophoretic elution (Towbin *et al.*, 1979). Two methods of electrophoretic transfer were used; *semi-dry* transfer, where the gel was sandwiched between absorbent paper soaked in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 20% (v/v) methanol, 0.0375% (w/v) SDS, pH 9.2) before being placed between electrodes, and *wet* transfer, where the gel sandwich was completely immersed in transfer buffer (25 mM Tris-HCl, 192mM glycine, 20% (v/v) methanol, pH 8.3) in a tank between platinum electrodes. Both techniques were used during the course of this work using Hoeffer apparatus. The manufacturer's instructions were followed throughout. The membrane used was Hybond-C (Amersham), a supported Nitrocellulose membrane.

2.16.1 Staining of membranes for total protein

After electrophoretic transfer, nitrocellulose membranes were stained in an aqueous solution of Ponceau S to reveal transferred protein and monitor blotting efficiency and completeness. A concentrated stock solution of Ponceau S was prepared (2% (w/v) Ponceau S in 30% (w/v) trichloroacetic acid) which was diluted ten times in deionized water before the immersion of the blot. The blot was allowed to stain for 5 mins before being washed gently in deionized water to remove unbound dye.

2.16.2 Generation of a polyclonal antiserum specifically against p64H1

Two adult New Zealand white rabbits were injected sub-cutaneously with 150 µl (at 1 mg/ml) of partially purified fusion protein (in this case a Protein A:BS2 fusion), in suspension with an equal volume of Freund's complete adjuvant. After 5 weeks, the rabbits received a "boost" immunisation of 150 µl of fusion protein mixed in suspension with an equal volume of Freund's incomplete adjuvant. This immunisation was repeated four more times at five-weekly intervals. One week after the final injection, the rabbits were sacrificed and blood removed. Serum (Ab 990) was prepared from the blood samples by incubating the samples at 37°C for 30 mins, followed by incubation on ice for 30 mins. The samples were placed at 4°C overnight to allow the clot to retract, and serum collected and stored in aliquots at -20°C before use.

2.16.3 Blocking and antibody incubations

Two blocking solutions were used in this study, and involved immersing the blot in either TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (w/v) Tween 20) containing 5% (w/v) BSA or 1 X PBS (50 mM sodium phosphate, pH 7.4, 150 mM NaCl) containing 5% (w/v) non-fat milk ("Marvel" powder) for at least 1 h at room temperature with gentle agitation. After blocking, the blocking solution was removed and the first antibody added, diluted in one of the above solutions. Incubation with the first antibody was for at least 1 h at room temperature with gentle agitation. After this incubation, the first antibody was removed, and the blots washed five times in large volumes of TBST or PBS for 5 mins each wash. After washing, the second antibody was added; either horseradish peroxidase conjugated donkey anti-rabbit IgG (SIGMA), diluted 1:15000 in blocking buffer, or alkaline phosphatase conjugated goat anti-rabbit IgG (Pierce), diluted 1:2000 in blocking buffer. Incubations with the second antibodies were for 1 h at room temperature, followed by extensive washing as before.

2.16.4 Detection of immune complexes on immunoblots

The second antibody conjugates used in this study demanded the use of two distinct detection methods: horseradish peroxidase conjugates were detected using an enhanced chemiluminescent reagent (ECL, Amersham), and alkaline phosphatase conjugates were detected using a NBT/BCIP mixed reagent system (Gibco BRL). Both methods were used according to the manufacturer's instructions.

2.17 Extraction of total RNA from rat tissue samples

The procedure used throughout this study was modified from that of Chomczynski and Saatchi (1987), based upon a guanidine thiocyanate/acid phenol protocol. All the total RNA extractions performed were as follows: Rat tissue (1 g) was freshly dissected from recently sacrificed SD rats and immediately placed in 9 ml of Solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% (v/v) Sarcosyl, 0.18% (v/v) β-mercaptoethanol) in an RNase-free Dounce-type homogeniser. The tissue was then homogenised with ten strokes of the homogeniser on ice. The homogenate was transferred to a RNase-free glass Corex tube, and the following additions made: 1 ml of 2 M sodium acetate, pH 4.0, 10 ml of acid phenol, water saturated, pH 4.5, and 2 ml of chloroform: isoamyl alcohol (24:1). The solutions were mixed together vigorously after each addition, and finally shaken vigorously for 15 s before incubation on ice for 15 mins. After this incubation, the mixture was centrifuged at 10 000 x *g* for 20 mins at 4°C. At this point, the RNA remained in the aqueous phase, with genomic DNA and proteins remaining in the organic phase. The upper phase was transferred to a fresh Corex tube, and the RNA precipitated by the addition of an equal volume of isopropanol, followed by incubation at -20°C for 1 h. The precipitated RNA was pelleted by centrifugation at 10 000 x *g* for 30 mins at 4°C. The resulting pellet was resuspended in 3 ml of Solution D, and precipitated as before. The final RNA pellet was washed once with RNase-free 70% (v/v) ethanol, briefly air-dried and resuspended in 500 µl of DEPC-treated water (deionized water, autoclaved for 30 mins after incubation with 0.05% (v/v) DEPC for 15 h).

2.17.1 Purification of poly $(A)^+$ RNA by affinity chromatography using oligo d(T) columns

Where mRNA was required to be purified during the course of this work, a commercial oligo d(T) column was used, the Poly(A) Quik system from Stratagene. The manufacturer's protocols were followed precisely.

2.18 Denaturing formaldehyde-agarose gel electrophoresis of RNA

Agarose gel electrophoresis of RNA was performed to assess the quality and quantity of extracted total and mRNA, and also as a prerequisite to Northern blotting. The method used was adapted from that of Sambrook *et al.*, (1989; pp 7.43-7.45). A 1% (w/v) agarose gel was prepared by melting agarose in 1 X FRB (formaldehyde running buffer, 20 X concentrate: 0.4 M MOPS, 0.1M sodium acetate, 0.02 M EDTA) by boiling. The gel was allowed to cool to approximately 55°C, when 17 ml of formaldehyde (37% (v/v)) were added, resulting in a final concentration of 2.2 M. The gel was cast and allowed to cool until firmly set. The RNA samples were prepared by incubating at 65°C for 15 mins in an equal volume of RNA Loading Buffer (50% (v/v) formamide, 1 X FRB, 2.2 M formaldehyde and 0.01% (w/v) bromophenol blue), loaded and electrophoresed at 5 V/cm in 1 X FRB, 2.2 M formaldehyde. Constant recircularisation of running buffer from the anode to the cathode prevented the generation of a salt gradient. After electrophoresis, the gel was rinsed briefly in DEPC-treated deionized water, and stained with ethidium bromide (0.5 μg/ml in DEPC-treated deionized water) for 15 mins. After staining, the gel was destained for 12 h in DEPC-treated deionized water.

2.18.1 Transfer of denatured RNA to Nylon filters (Northern blotting)

If the RNA was to be blotted, the staining step was omitted. The transfer of denatured RNA to nylon filters was carried out as described in Sambrook *et al.*, (1989; pp 7.39-7.52). After transfer, the RNA was cross-linked to the Nylon filter using UV-light in a Stratalinker, according to the manufacturer's instructions (Stratagene). The transfer efficiency was monitored by cutting a spare lane from the blot and staining with methylene blue (Sambrook *et al.*, 1989; p 7.51).

2.19 Northern hybridisation

Every Northern hybridisation, and Southern hybridisation (including plaque-lift hybridisation) performed during the course of this study used the following protocol. The filter was pre-hybridised for 2 h at 65°C in hybridisation buffer (6.25 ml 20 X SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA), 2.5 ml 50 X Denhardt's solution (5% (w/v) Ficoll, 5% (w/v) polyvinylpyrollidine, 5% (w/v) BSA), 200 µl single-stranded salmon sperm DNA (10 mg/ml), 625 µl 20% (w/v) SDS, 11.5 ml DEPC-treated deionized water), in a rolling bottle hybridisation oven (Techne). Labelled probe was added, and hybridised for 15 h at 65°C. Washing conditions varied with the level of stringency required for each particular experiment: low stringency washes (2 X SSC, 65°C, 15 mins), followed, if required by moderate stringency (2 X SSC, 0.1% (w/v) SDS, 65°C, 30 mins), followed, if required by high stringency (0.2 X SSC, 65°C, 10 mins). After the final wash, the filters were wrapped in Saran wrap and autoradiographed.

2.20 mRNA *in situ* Hybridisation

2.20.1 Preparation of cryostat sections

Whole brains were dissected from freshly sacrificed adult SD rats and immediately placed on a piece of aluminum foil on dry ice to freeze slowly. The frozen brain samples were mounted on the pre-cooled cryostat cutting block with Tissue-Tek (Miles Laboratories). After mounting, the sections were allowed to equilibrate to the sectioning temperature in the cryostat chamber for 1 h at -12°C. Serial sections were cut at a thickness of 12-15 μm and thaw mounted onto sterile, RNase-free microscope slides subbed with poly-L-lysine (0.01% (w/v) in DEPC-treated water)

2.20.2 Fixation and preparation of sections for hybridisation

The mounted sections were allowed to air-dry for 30 mins before processing. After this time, the sections were fixed in 4% (w/v) phosphate-buffered paraformaldehyde (4% (w/v) paraformaldehyde in 1 X PBS (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4)) for 20 mins on ice, followed by a brief wash in 1 X PBS, and dehydration through graded alcohols (1 minute each in 40% (v/v), 60% (v/v), 80% (v/v), 95% (v/v) and 100% ethanol). The sections were then acetylated to reduce non-specific probe binding and remove excess lipid material (Young *et al.*, 1986) by rehydrating briefly in 1 X PBS, then incubating in acetylation solution (0.25% (v/v) acetic anhydride in 0.1 M triethanolamine-HCl, pH 8.0/0.9% (v/v) HCl) for 10 mins at room temperature. The sections were then dehydrated through graded alcohols as before, before delipidation in 100% chloroform for 5 mins at room temperature.

After these treatments, the sections were again dehydrated by passing through graded alcohols, and stored in 95% (v/v) ethanol at 4°C until required for hybridisation.

2.20.3 Preparation of end-labelled oligonucleotide probes by terminal deoxynucleotidyl transferase (TdT) labelling

A 45-mer antisense oligonucleotide probe was used in *in situ* hybridisation experiments. The following were assembled in a sterile, RNase-free 1.5 ml tube: 1.25 µl 10 X tailing buffer (200 mM sodium cacodylate, pH 7.2, 4 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5 mg/ml BSA), 8.25 µl DEPC treated deionized water, 1 µl 5'-[α^{35}S]-dATP (12.5 µCi/µl), 1 µl TdT (20 U/µl, Gibco BRL). The reaction mixture was incubated at 30°C for 1 h. The reaction was terminated by the addition of 40 µl of DEPC-treated deionized water.

2.20.4 Purification of tailed probes with Sephadex G-25 spin columns

Sephadex G-25 spin columns were prepared described elsewhere (Sambrook *et al.*, 1989; pp E37-E38).

2.20.5 Analysis of tailing reaction by denaturing polyacrylamide gel electrophoresis

After the tailing reaction and Sephadex G-25 purification, an aliquot of the labelled probe (5 µl) was electrophoresed in a 6% denaturing polyacrylamide gel.

2.20.6 Hybridisation of probe to tissue sections

Just prior to hybridisation, the sections were removed from 95% (v/v) ethanol and allowed to air-dry for 15 mins. The following hybridisation buffer was prepared in a 50 ml sterile polypropylene tube:

10% formamide	25 ml
20 X SSC	10 ml
0.5 M sodium phosphate, pH 7.0	2.5 ml
0.1 M sodium pyrophosphate	0.5 ml
50 X Denhardt's solution	5 ml
denatured salmon sperm DNA (10 mg/ml)	1 ml
polyadenylic acid (5 mg/ml)	1 ml
heparin (120mg/ml)	50 µl
dextran sulphate (Pharmacia)	5 g

DEPC-treated deionized water was added to a final volume of 50 ml. The tailed probe was diluted 1:100 in the hybridisation buffer, and 20 µl of 1 M DTT were added. Sixty microlitres of probe/hybridisation buffer was applied to each section and sealed with Parafilm. The sections were placed in a sealed chamber humidified with 2 X SSC/50% (v/v) formamide and incubated at 42°C for 15 h.

2.20.7 Washing and autoradiography of *in situ* hybridisations

After hybridisation, the Parafilm covers were carefully removed. The sections were washed briefly in 1 X SSC at room temperature, then washed in pre-warmed 1 X SSC for 30 mins at 55°C. After this washing step, the sections were passed briefly

through 1 X SSC, 1 X SSC/70% (v/v) ethanol, and 95% (v/v) ethanol, all at room temperature. The sections were allowed to air-dry for 30 mins before being exposed to X-ray film for 10-20 days at room temperature.

2.21 Polymerase Chain Reaction (PCR)

PCR was used extensively throughout this work: the cloning of p64H1 followed a reverse-transcriptase PCR strategy (e.g. Lynas *et al.*, 1989; Chelly *et al.*, 1988); RACE (and cRACE) strategies were used to isolate cDNA clones from unknown mRNA sequences (Frohman *et al.*, 1988; Maruyama *et al.*, 1995); PCR was used as a tool for analyzing transformants after cloning experiments (Gussow *et al.*, 1989), and it was used in creating suitable restriction endonuclease sites (Clackson *et al.*, 1994) to aid the generation of cDNAs encoding fusion proteins. Individual thermal cycling conditions for each PCR are described in the following chapters as appropriate.

2.22 Yeast two-hybrid library screening

Approximately 5 x 10^6 independent library clones were screened for interaction with the fusion protein encoded by pAS2-H1 according to the manufacturer's instructions. The *S. cerevisiae* strain CG1945 was simultaneously transformed with pAS2-H1 and the library plasmids then plated on SD-Trp/-Leu/-His/+ 5 mM 3-AT and incubated at 30°C for 5 days until colonies could be seen. Putative positive colonies were re-plated on the same medium and grown again as before. The colonies were then

screened for activation of the *LacZ* reporter using a β-galactosidase assay according to the manufacturer's instructions.

2.22.1 Preparation of yeast protein extracts for Western blot analysis

Total protein extracts were prepared from transformed yeast clones in order to study the expression of GAL 4 DB-fusion proteins. A single transformed colony grown on selective media (SD -Trp) was picked and cultured in 10 ml of SD -Trp media at 30°C overnight with shaking (225 rpm). The entire overnight culture was used to inoculate 50 ml of YPD media, and cultured as before until the yeast entered exponential growth. The culture was immediately cooled, and then poured into a centrifuge bottle containing 25 ml of ice. The yeast were collected by centrifugation at 1000 x *g* for 5 mins, then resuspended in 50 ml of sterile water and centrifuged again. The pellet was resuspended in 300 µl of yeast cracking buffer (8 M urea, 5 % (w/v) SDS, 40 mm Tris-HCl, pH 6.8, 0.1 mM EDTA, 1% (v/v) β-mercaptoethanol, 1 X protease inhibitor solution (Complete™ tablets, Boehringer Mannheim)), and incubated at 60°C for 10 mins. Eighty-microlitres of glass beads (425-600 µm, Sigma) were added, and the mixture vortexed vigorously for 1 minute. The mixture was centrifuged at 14 000 rpm for 5 mins, and the supernatent retained for analysis. Control samples were prepared in a similar way, using untransformed strain CG1945, cultured in SD -Ura media.

2.23 Mammalian cell culture

HEK293 cells (transformed human embryonic kidney cell-line) and HT4 cells (transformed rat neuronal stem-cell fusion) were cultured in DMEM (Gibco BRL) supplemented with 10% (v/v) foetal calf serum (Gibco BRL), 2 mM L-glutamine (Gibco BRL), and 2 mM sodium pyruvate (Gibco BRL). Cells were cultured and passaged according to standard procedures (Doyle *et al.*, 1994).

2.23.1 Transfection of mammalian cells

During the course of this study, calcium phosphate-mediated transfection and liposome-mediated transfections were performed. Reagent kits were purchased from Promega (CalPhos Profection kit and Tfx-20 lipid reagent, respectively) and the manufacturer's instructions followed precisely.

2.24 Indirect immunofluorescence

Cells to be used in immunofluorescence studies were cultured in 30 mm tissue culture dishes containing a sterile, 10 mm^2 glass coverslip. This permitted the transfer of cells adhered to the coverslip to other vessels for fixation and antibody binding. Cells were transfected (if appropriate) 48 h before immunofluorescence was to be performed. The culture media was removed from the cells by aspiration. The cells (adhered to the coverslip in a dish) were washed twice with 1 X PBS before being immersed in fixative (4% (w/v) buffered paraformaldehyde, pH 7.0) for 20 mins. This solution was removed after fixation by aspiration, and the fixed cells again washed twice with PBS. After this washing step, the cells were incubated for

10 mins in a solution of 1 X PBS/50 mM NH_4Cl to quench potential autofluorescence. The cells were again washed twice in 1 X PBS, then placed in 2 ml of 0.1% (v/v) Triton X-100 in 1 X PBS to permeabilise the cells. After two more washes as before, the coverslips were inverted onto 100 µl of antibody (polyclonal antiserum, diluted in 1:100 in 1 X PBS, 0.2% (v/v) fish skin gelatin) and incubated at room temperature for 1 h. The coverslips were washed three times in 1 X PBS, 0.2% (v/v) fish skin gelatin before being inverted onto 100 µl of fluorochrome-labelled second antibody (rhodamine conjugated anti-rabbit IgG, diluted 1:100 in 1 X PBS/0.2% (v/v fish skin gelatin) and incubated for a further hour at room temperature. The coverslips were then washed three times in 1 X PBS, 0.2% (v/v) fish skin gelatin and twice in 1 X PBS. If required, the cells were stained with Hoechst dye number 33258 (2 µg/ml in 1 X PBS) for 20 mins, after which they were washed three times in 1 X PBS. Some cells were also stained with the dye Di-O-$C_5(3)$, by placing in a solution of 1 µg/ml of dye in 75% (v/v) methanol for 20 mins at room temperature, after which they were washed three times in 1 X PBS. The coverslips were mounted using 20 µl of Movoil (BDH) and allowed to set for 6 h before examination using a Leitz Ortholux Fluorescence microscope with a standard FITC/rhodamine block. Photographs were taken using a Leitz Orthomat camera controller.

2.25 Immunohistochemistry

Rat brain sections were prepared using a cryostat as before (Chapter 2.20.1), and fixed for 10 mins in 4% (w/v) buffered paraformaldhyde at room temperature. Immunoreactive protein was detected using Ab990 and the ExtraAvidin™ peroxidase staining kit (SIGMA). The manufacturer's instructions were followed throughout.

2.26 Preparation of microsomal membranes

Microsomal membranes were prepared from rat tissue and cultured cells as follows: the sample was washed briefly in cold Homogenisation Buffer (0.32 M sucrose, 5 mM Tris-HCl, pH 7.4; 1 X solution of antiproteases (Boehringer Mannheim Complete™)), before homogenisation/cell lysis in a Dounce-type glass-teflon homogeniser. Differential centrifugation was used to separate the P1 (1000 x *g*, 15 mins), P2 (3000 x *g*, 10 mins) and P3 microsomal (100 000 x *g*, 30 mins) fractions. The pellets were resuspended in small volumes of Homogenisation Buffer and stored at -80°C.

Chapter 3

Isolation of a clone encoding *p64H1*, a homologue of p64

3.1 Introduction

The isolation of a cDNA clone encoding *p64H1* followed what is now a classical route: a partial cDNA was amplified using fully degenerate PCR primers, followed by cDNA library screening and RACE-PCR. The combination of these procedures allowed the isolation of a cDNA derived from rat brain mRNA which encoded the entire open reading frame of a previously undescribed protein.

3.2 Introduction to PCR

3.2.1 Deoxynucleotide primer considerations

The success or failure of any PCR experiment depends in part upon the design of the primers to be used in the amplification procedure. Generally, PCR primers were between 15-25 bases in length, with approximately equal GC and AT content. Pairs of primers used in a particular amplification experiment ideally possessed similar melting temperatures. The ability of a particular primer to form "hairpins" (i. e. form secondary structure through self-complementarity) was undesirable, as this impairs primer annealing and subsequent amplification. Likewise, regions of complementarity between primers in the same reaction were avoided because of the likelyhood of hybrid formation ("primer-dimer") interfering with the amplification. Primer sequences need not match that of the template exactly; site-directed mutagenesis incorporating changes designed into the primer sequence is a commonly used technique (Clackson *et al.*, 1994). Recognition sequences for particular restriction endonucleases may also be incorporated into the primer, facilitating subsequent cloning experiments. This strategy was used in the following

experiments. In all cases where there was likely to be some mis-match between the primer sequence and the template DNA sequence, these were incorporated near the 5'-end of the oligonucleotide. Mis-matches near the 3'-end of the oligonucleotide de-stabilise the primer-template hybrid in this region, so that extension is very inefficient or non-existent (Sommer and Tautz, 1989). In addition to the above considerations, primer sequences may be degenerate, in which case a mixture of primers encoding every possible codon variation is used. In this way, a primer sequence may be designed from a back-translated amino acid sequence, and used to amplify cDNAs encoding closely-related proteins. Such degenerate oligonucleotides may frequently require up to six variants at particular site; at these positions, a universal nucleotide (inosine) which base-pairs with all of the other bases was used (Knoth *et al.,* 1988). This reduced the level of redundancy and the subsequent complexity of the primer mixture.

3.2.2 Reverse-transcription PCR (RTPCR)

The thermostable polymerases used in the PCR process are DNA dependent in that they require a DNA template in order to direct the synthesis of a complementary strand of DNA. Although some thermostable polymerases possess some reverse-transcriptase activity (e. g. *Taq* polymerase, Saiki *et al.,* 1988), this activity is very limited. In many cases, the amplification of RNA from a tissue or organism would be desirable. In these examples, a reverse transcriptase is used to direct first-strand cDNA synthesis from an RNA template. The reverse transcription may be directed from a gene-specific primer, random hexadeoxynucleotides, or an oligo d(T) primer.

Using a gene-specific primer has the advantage of increased specificity in a subsequent amplification reaction, but often generates very low yields of first-strand cDNA. In addition, some gene-specific primers may not work efficiently due to regions of secondary structure within the mRNA template over the annealing site. Random hexadeoxynucleotides (random primers; Koike *et al.*, 1987) initiate cDNA synthesis from internal sites within the mRNA molecule. Random primers can be used to synthesise a mixed population of cDNAs representing regions of a particular mRNA possessing strong secondary structure. Finally, an oligo d(T) primer anneals to the 3'-poly(A) tail of eukaryotic mRNA, therefore cDNA synthesised in this manner represents the 3'-regions of mRNA (Sambrook *et al.*, 1989).

3.2.3 Reverse transcriptase considerations

Two reverse transcriptase enzymes are commonly used: Moloney murine leukaemia virus (MMLV) and avian myoblastoma virus (AMV) reverse transcriptases. Both these enzymes may be used successfully in RTPCR reactions, but MMLV has been demonstrated to be more efficient at generating long cDNA species (Hu *et al.*, 1991). In addition, these enzymes possess an intrinsic RNase H activity which will selectively degrade RNA which is part of an RNA:DNA hybrid, so reducing the amount of RNA available for subsequent reverse transcription. A modified, recombinant MMLV reverse transcriptase is available which has been engineered to remove the RNase H activity (Superscript II, GIBCO BRL) so increasing first-strand cDNA yields in a reverse transcription reaction. This enzyme was the reverse transcriptase used during this study. It has been suggested that the presence of the

RNA transcript in the subsequent PCR amplification can reduce the reaction efficiency by competing with the cDNA for the gene-specific oligonucleotides, especially in reactions where an RNase H$^-$ reverse transcriptase has been used (Hu *et al.*, 1991), necessitating the removal of the RNA by RNase H treatment or alkali hydrolysis before amplification. This was not found to be required in RTPCR experiments in this work. Several cDNA synthesis reaction mixes have been designed for compatibility with the PCR reaction mix (e. g. Chelly *et al.*, 1988) allowing cDNA synthesis and PCR amplification to be carried out in the same tube. This eliminated some potential sources of contamination associated with extra handling steps. A single tube RTPCR protocol was used in this project.

3.2.4 Thermal cycle conditions

Every PCR experiment is unique and is differentiated by differences in thermal cycle conditions. The thermal cycle can be divided into three distinct steps: (1) a denaturing step, incubating the reaction mixture at 94-95°C to denature the double-stranded template; (2) an annealing step of varying temperature to allow the primers to anneal to their target sequences; and (3) an extension step, at the optimum temperature for thermostable polymerase activity (e. g. 72°C for *Taq* polymerase). The incubation times at each step may be kept to a "standard" for PCR: 1 minute for denaturation, 1 minute for annealing, and 1 minute for each kilobase of product to be amplified. In practice, these times may be greatly reduced in order to reduce the time required for amplification. The number of cycles used may also be kept constant: 30 cycles was most common. The variable temperature step (the annealing

stage) depended upon the melting temperature (T_m) of the particular primers used in each experiment. The T_m may be defined as the temperature at which half of the primer molecules remain annealed to their complementary sequences, and may be roughly calculated using the following equation: [2 x (A + T)] + [4 x (C + G)] (Sambrook *et al.*, 1989). An accurate value for the T_m of a deoxyoligonucleotide may be obtained using the following equation:

$$T_m = 81.5 + 16.6(\log_{10} [Na^+]) + 41.0 \text{ (fraction C + G)} - (600/N)$$

where N = deoxyoligonucleotide length in bp (Bolton and McCarthy, 1962). Non-specific amplification may arise due to primer annealing before the amplification reaction starts: this may be circumvented by omitting one essential component of the reaction mixture until the thermal cycle starts ("hot-start"; D'Aquila *et al.*, 1991). In every PCR amplification in this study, the addition of the thermostable polymerase was withheld until the samples had been incubated at 95°C.

3.2.5 Thermostable polymerase considerations

DNA synthesis by DNA polymerases is a highly ordered and complex molecular process, during which a deoxynucleotide triphosphate (dNTP) substrate is added to a 3'-hydroxyl of a DNA primer-template (reviewed by Kuchta *et al*, 1988). At least three opportunities exist for a DNA polymerase to discriminate against errors (Eckert *et al.*, 1990). The first step in error discrimination is the ability of the DNA polymerase to distinguish among incoming dNTP substrates and to incorporate the correct dNTP on to a DNA-primer template. Different DNA polymerases differ in their ability to distinguish between dNTP substrates, and 10- to 100- fold variations

in base substitution error rates are reported (Mendelman *et al.*, 1989; Bebenek *et al.*, 1990). The second step, the extension step, is the selective ability of a particular DNA polymerase to continue synthesis on a correctly paired primer-template as opposed to a primer-template containing a terminal mis-match. As before, the rates of extension for any particular polymerase can vary widely (Bebenek *et al.*, 1990; Mendelman *et al.*, 1989). Finally, some, but not all DNA polymerases possess a 3'-5' exonuclease activity, which can selectively remove mis-incorporated nucleotides to regenerate a correctly base-paired primer-template terminus ("proof-reading"; reviewed by Kunkel and Bebenek, 1988). The error rate of a DNA polymerase *in vitro* is not a constant, but depends upon the conditions of the reaction and the particular enzyme used. The number of cycles, dNTP concentration, buffer used and the denaturation conditions used (extended incubation at 95°C can cause de-purination of the template DNA, (Barnes, 1994)) can all affect polymerase fidelity (Eckert and Kunkel, 1990).

3.2.6 Buffer considerations

Magnesium (Mg^{2+}) concentration is a crucial factor affecting the performance of *Taq* and other DNA polymerases. Reaction components, including the template DNA, chelating agents present in the sample (e. g. EDTA), dNTPs and proteins all affect the concentration of free Mg^{2+}. In the absence of adequate Mg^{2+}, *Taq* polymerase is completely inactive (Saiki *et al.*, 1988). Conversely, excess free Mg^{2+} reduces enzyme fidelity (Eckert and Kunkel, 1990) and may increase the likelyhood of non-specific amplification (Ellsworth *et al.*, 1993). All of the PCR experiments in this

work used a final concentration of total Mg^{2+} of 2.5 mM. Several reaction buffer formulations have been published, but a consensus has emerged which is similar to that described by Saiki *et al.*, in 1988. This contained Tris at a final concentration of 10 mM (pH 8.4 at 20°C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.01% (v/v) Nonidet P40, and 0.01% (v/v) Tween 20. The non-ionic detergents may be replaced with 0.1% (v/v) Triton X-100, but some detergent is essential in the reaction buffer to maintain enzyme activity. The reaction buffer used in PCR experiments here was supplied by Promega and contained 50 mM KCl, 10 mM Tris-HCl, pH 9.0 (at 25°C) and 0.1% (v/v) Triton X-100; all final concentration. This buffer lacks Mg^{2+} which was added separately from a 25 mM stock solution of $MgCl_2$.

3.2.7 "Long Distance" PCR (LDPCR)

It has traditionally been assumed that PCR is intrinsically limited in that the maximum template length which can be amplified is around 5 kb. In general, the thermostable polymerases used in PCR amplifications do not possess "proofreading" activity (3'-5'- exonuclease activity; e. g. *Taq* polymerase). The reasons behind the inefficiency of long amplifications were analysed in three papers (Barnes, 1994; Cheng *et al.*, 1994 a; 1994 b) where conditions were laid out that could allow for amplification of DNA up to 40 kb. The dNTP concentration, buffer concentration and cycle conditions were optimised for LDPCR, but perhaps the most interesting finding was that the polymerase used had a profound effect upon amplification efficiency. Both papers reported that using a cocktail of thermostable polymerases

consisting mainly of *Taq* polymerase, but supplemented with a polymerase with "proofreading" ability (e. g. *Pwo* polymerase) allowed for the most efficient amplification. The second polymerase has the ability to recognise and correct primer-template terminal mis-matches, so allowing *Taq* polymerase to continue primer extension (Barnes, 1994; Cheng *et al.*, 1994 a, b). The LDPCR system used in this study was the Expand™ Long-Range PCR system (Boehringer Mannheim), which used a proprietary cocktail of *Taq* and *Pwo* polymerases.

3.3 Results

3.3.1 Amplification of a partial cDNA, *BS2*, from rat brain using degenerate RTPCR

Two fully degenerate PCR primers were designed based upon the amino acid sequence of p64 (Landry *et al.*, 1993; GenBank Accession number L16547). The sequence regions chosen were contained within two of the putative transmembrane regions of p64. In order to avoid the level of codon redundancy required for amino acids with six possible codons (e. g. serine, arginine and leucine), the universal base inosine was used (Knoth *et al.*, 1988; Figure 3.1). In addition, the sequence of the "forward" primer was chosen to encode an amino acid sequence containing a methionine residue and a tryptophan residue, the only amino acids which have a single codon possibility. In order to facilitate the cloning of PCR products amplified using these oligonucleotides, a terminal (5'-) sequence was included in each primer encoding the recognition site for *Eco*R I. An additional 3 bases 5'-to these sequences were included to increase the digestion efficiency of the restriction enzyme: the efficiency of restriction digestion at the ends of double- and single-stranded DNA

M3044: **F** **M** **I** **L** **W**

TT(T/C) ATG AT(T/C/A) TT(G/A)/ TGG

CT(T/G/C/A)

TTY ATG ATH YT*I* TGG

5'-CAC<u>GAATTC</u>TTYATGATHYT*I*TGG-3'

M3045: **D** **G** **D** **E**

GA(T/C) GG(A/T/C/G) GA(T/C) GA(G/A)

CTR CCX CTR CTY (complementary)

YTC RTC *I*CC RTC (reverse)

5'-CAC<u>GAATTC</u>AAYTCRTC*I*CCRTC-3'

Figure 3.1 The sequences of primers M3044 and M3045. The *Eco*R I sites are underlined. The deoxyinosine residues are represented by the symbol *I*. The reverse primer M3044 contains an extra two adenosine residues, corresponding to the reverse complement of the first two bases of the next codon.

molecules is often greatly reduced when compared to that of recognition sites contained within polynucleotides (Moreira and Noren, 1995). The first-strand cDNA synthesis was directed from the degenerate gene-specific reverse primer rather than an oligo-d(T) primer to increase the probability that only the desired cDNA was synthesised. The RTPCR was performed as follows. Total RNA was extracted from rat brain, and an aliquot (1 μg) electrophoresed in a 1% (v/v) denaturing formaldehyde agarose gel to confirm recovery and quality (Figure 3.2, a). One microgram of total RNA was used in the reverse-transcription reaction as follows;

$MgCl_2$ (25 mM)	2 μl
10 X PCR buffer (Promega)	2 μl
dNTP mixture (10 mM each)	2 μl
RNasin (40 U/μl)	0.5 μl
"reverse" primer M3045 (10 pmoles/μl)	2 μl
total RNA (0.5μg/μl)	2 μl
RNase-free water	8.5 μl

This reaction mixture was incubated at 70°C for 10 mins to denature mRNA secondary structure, and allowed to cool slowly to 23°C to permit primer annealing. Ten units (1 μl) of Superscript II MMLV-RT were added and the reaction mixture incubated at 42°C for 45 mins, before a 5 minute incubation at 95°C to terminate the reaction and denature the cDNA-RNA hybrids. Immediately following this reaction, the PCR components were added;

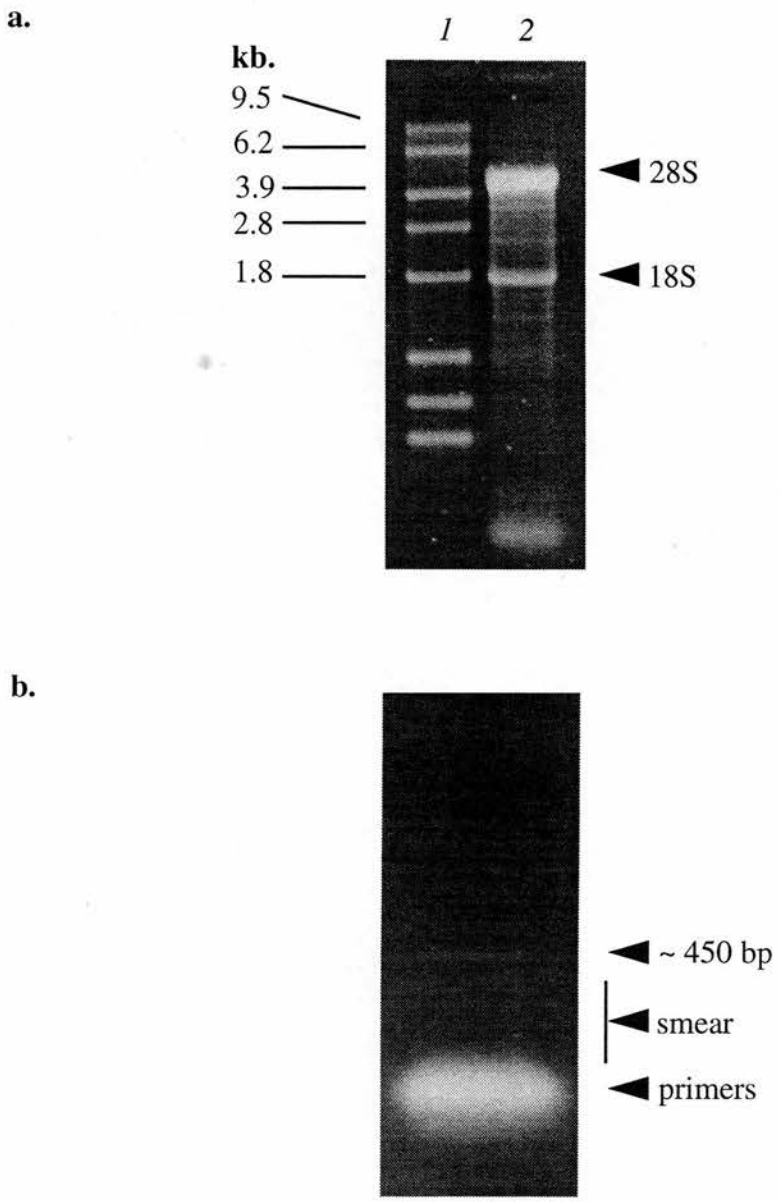


Figure 3.2 Rat brain RTPCR. a. One microgram of total RNA extracted from rat brain and electrophoresed in a 1% (w/v) denaturing agarose-formaldehyde gel, stained with ethidium bromide. Lane 1: RNA markers (Sigma). Lane 2: Total RNA. **b.** RTPCR amplified products from rat brain total RNA. The band and the lower smear were excised separately.

$MgCl_2$ (25 mM)	8 µl
10 X buffer (Promega)	8 µl
"forward" primer M3044	
(10 pmoles/µl)	1 µl
"reverse" primer M3045	
(10 pmoles/µl)	1 µl
sterile deionized water	62.5 µl

The thermal cycling conditions were as follows; 95°C, 1 minute, after which 0.5 µl of *Taq* DNA polymerase were added, followed by 95°C, 30 seconds; 37°C, 30 seconds; 72°C, 2 mins; 30 cycles. An aliquot (5µl) of the PCR was electrophoresed in a 1% (w/v) agarose gel to visualise the amplified products (Figure 3.2, b). A parallel, negative control experiment was also performed. This reaction was exactly as the test experiment, but 1 µg of total RNA was added instead of cDNA to test the RNA preparation for genomic DNA contamination. This reaction produced no products. Two species were excised from the gel and purified using a QIAEX II kit. These species were designated B (band), S (smear). An aliquot (1 µl) of each of the gel-purified DNA pools was re-amplified using the same primers with an increased annealing temperature (50°C), and the products analysed as before using agarose-gel electrophoresis (Figure 3.3). Three re-amplified PCR products (B, BS1 and BS2) were purified and cloned into the *Eco*R I site of pBluescript II KS (Appendix I) and pGEM-T (Appendix I) and used to transform XL1-Blue cells. The resulting transformants were analysed and recombinant plasmids were propagated and their inserts sequenced from vector-encoded sites. The clone obtained from the

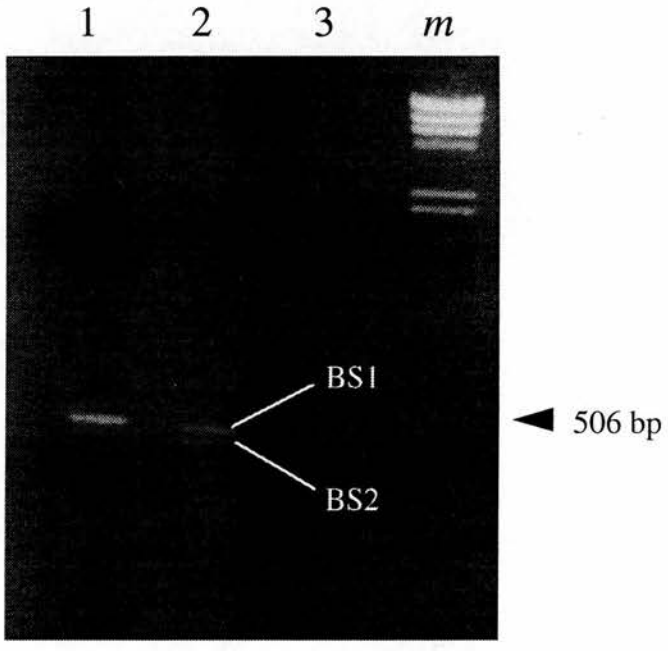


Figure 3.3 RTPCR re-amplified products from rat brain mRNA.

Agarose gel electrophoresis of re-amplified "band" and "smear" DNA.

Lane 1: "Band"; Lane 2: "Smear"; Lane 3: no DNA control; *m:* 1 kb markers (Gibco BRL). The 506 bp marker is indicated.

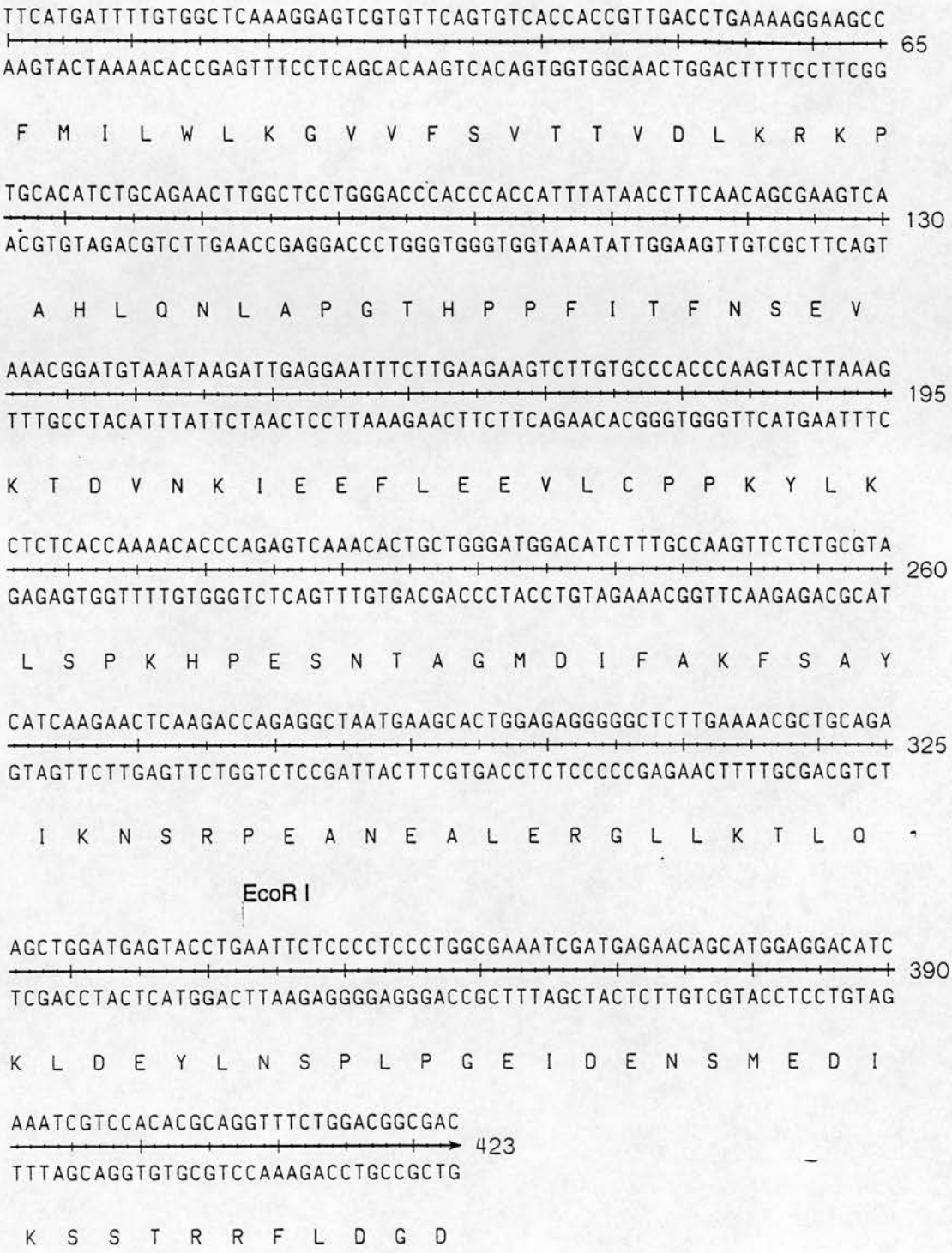


Figure 3.4 The nucleotide and deduced amino acid sequences of *BS2*.

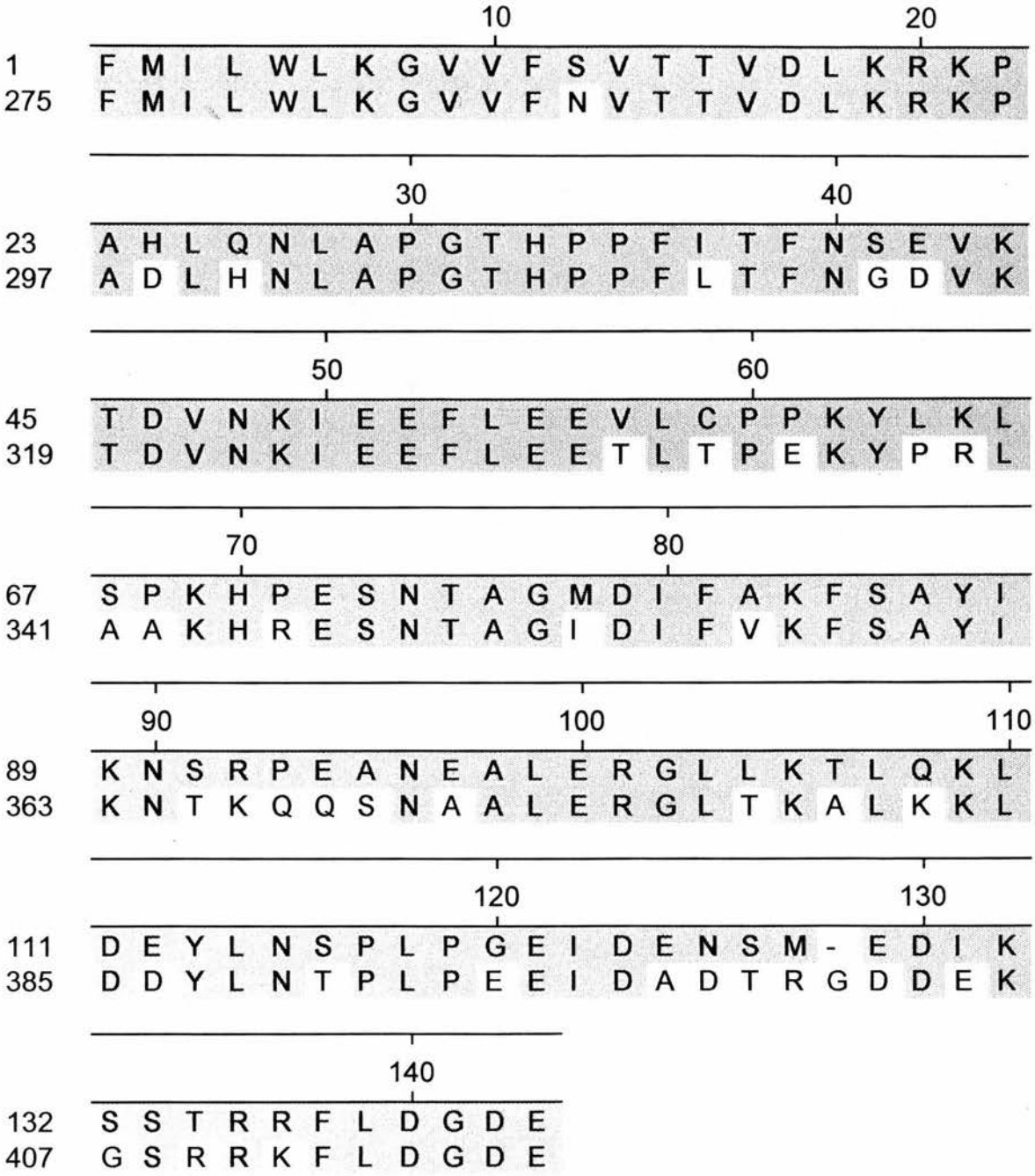


Figure 3.5 An optimal alignment between bovine p64 (top) and the deduced amino acid sequence of *BS2*. Alignments were performed using the Clustal algorithm of the MegAlign program within the DNAStar package. Residues matching BS2 are shaded in yellow. The identity is 71%.

to the published nucleotide sequence for bovine p64, and approximately 74% identity to the amino acid sequence for bovine p64. The sequence data obtained is presented in Figure 3.4. This clone was named *BS2* (Brain Smear 2). The data obtained from the other clones (B and BS1) were identical and showed no similarity to any sequence contained within the GenBank database. An optimal alignment of *BS2* with *p64* is shown in Figure 3.5. The sequence contained an extra recognition site for *Eco*R I at position 342 (position number relative to the first base of oligo M3044) not contained within the nucleotide sequence of bovine p64, revealed by sequencing the plasmid pGEM-T.BS2, generated by ligating *BS2* into the T/A-type vector GEM-T. The 346 bp *Eco*R I restriction fragment excised from the plasmid pBluescript II.BS2 was used in subsequent experiments as a probe in attempts to isolate a clone from cDNA libraries which encoded the entire open reading frame of the rat brain homologue of p64.

3.3.2 Further work towards the isolation of a clone encoding the entire ORF of the rat brain homologue of p64

Rapid Amplification of cDNA ends-PCR was the chosen technique for the isolation of the regions of cDNA flanking *BS2*. This procedure, originally described by Frohman (1994), has been used successfully in numerous cases to isolate and clone cDNA from regions of unknown sequence flanking partial cDNA clones (e. g. Lalioti *et al.*, 1997; Malinowska *et al.*, 1995). The attraction of such a technique over conventional cDNA library screening are severalfold: the method is rapid, and potentially permits the cloning of flanking regions in a matter of days. RACE-PCR

is less expensive than library screening and does not require the use of hazardous radioisotopes. Unfortunately, despite a thorough attempt at optimising the conditions required for the specific amplification of both 5'- and 3'-RACE-PCR products, a single PCR product was never obtained. The requirements for such a specific product are numerous; excellent quality mRNA is required as a template for first-strand cDNA synthesis, and the reverse transcriptase is required to synthesise long, if not complete, cDNA. The chosen oligonucleotide primers to direct cDNA synthesis must anneal efficiently to the mRNA template, despite potentially extensive mRNA secondary structure. This condition is difficult to fulfil as some primer sequences may never efficiently anneal to their template. In addition, the "tailing" reaction of the first-strand cDNA required when attempting to isolate regions 5' to the known sequence is difficult to control, and terminal deoxynucleotidyl transferase may catalyse the addition of variable numbers of deoxynucleosides to each cDNA in a particular sample. Finally, the conditions required to optimise any particular PCR are complicated and many-fold, and this is made even more problematic when one of the primers required is "universal" in nature.

After unsuccessful attempts to amplify regions of cDNA flanking *BS2*, it was decided to use a conventional cDNA phage library screening technique to isolate a full-length clone.

3.3.3 Screening a cDNA phage library

Bacteriophage lambda (λ) has been used as a cloning vector for many years (e. g. Frischauf *et al.*, 1983; Young and Davis, 1983). Several years of extensive genetical scrutiny has allowed the development of several excellent λ phage vectors and associated bacterial strains to permit the cloning of recombinant DNA and cDNA (e.g. Short *et al.*, 1988; Bullock *et al.*, 1987). The advantages of using λ phage vectors for the construction of cDNA libraries include the capacity for cloning large inserts, but most importantly the extremely high packaging (and subsequent transformation) efficiency that phage vectors afford. Two λ phage cDNA libraries were screened during the course of this study; an oligo d(T)-primed rat olfactory bulb cDNA library constructed in the vector λZAP II (Stratagene), and a random-primed and oligo d(T)-primed rat whole brain cDNA library constructed in the vector λgt 10 (Clontech).

3.3.4 Screening of a cDNA library constructed in λZAP II

The titre of the library was determined, and found to be approximately 1.2×10^9 pfu/ml. Approximately 1×10^6 independent clones from this library were screened using an [α^{32}P]-dCTP labelled probe, derived from the *Eco*R I restriction fragment of *BS2*. After a single round of screening at high stringency, 20 putative positive plaques were isolated, and their titres after an overnight incubation in SM buffer determined. After a second round of screening using the same probe and conditions, 13 of the clones from the primary screen were confirmed positive, and these plaques were isolated and phagemid DNA rescued using the *in vivo* excision feature of λZAP

II. Vectors of the λZAP group have the enormous advantage of featuring *in vivo* excision of phagemid DNA containing the cloned cDNA insert (Bullock *et al.*, 1987; Short *et al.*, 1988). This *in vivo* excision depends upon DNA sequences placed in the λ phage genome, and the presence of several *trans*-acting proteins derived from a filamentous phage. The filamentous phage proteins recognise a region of DNA normally serving as the f1 filamentous phage "origin of replication" for positive strand DNA synthesis. The origin of replication may be sub-divided into two overlying components: (1) the site of initiation, and (2) the site of termination of synthesis. These two regions have been sub-cloned individually into the λZAP vectors, flanking the sequences of a phagemid vector (in the case of λZAP II this was pBluescript II SK).

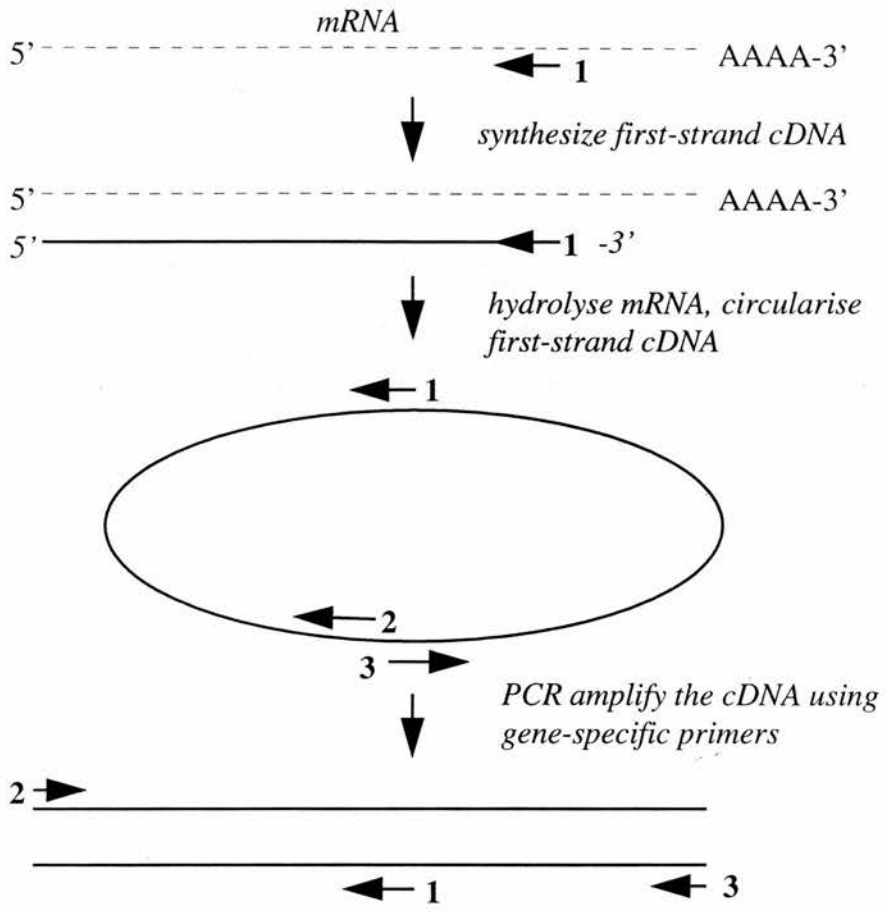
Inside *E. coli,* the "helper" proteins derived from the filamentous phage recognise the initiator DNA within the λZAP sequence. One of these proteins then nicks one of the vector strands, where new DNA synthesis begins, continuing until the terminator sequence is encountered. The single-stranded DNA molecule is circularised by the gene II product from the filamentous phage, and this molecule is packaged and secreted from the *E. coli* cell. Once the phagemid is secreted, the host cells can be killed by heating to 70°C. Another host strain of *E. coli* (in this case the *SOLR* strain was used) may then be introduced to propagate the excised phagemid. Sequence analysis of the phagemid DNA, using the vector-derived T7 and T3 primers, revealed that none of the 13 clones contained full-length cDNA. All the clones obtained were identical, and all contained sequence that was identical (where overlapping) to *BS2*. None of the clones obtained contained cDNA that extended

further upstream than *BS2*, although all the clones contained extensive 3'-flanking sequence. It was deduced that the mRNA from which *BS2* was derived contained an unusually long 3'-untranslated region (UTR) of approximately 4 kb. Further sequence analysis of the flanking coding region of these clones allowed the determination of all the sequence from the original forward primer of *BS2* (M3044) to the 3'-end of the coding region. The deduced amino acid sequence from this data revealed the sequence to be approximately 71% identical to bovine p64, and that both predicted sequences contained termination codons in similar relative positions. No consensus sequences for glycosylation were found to be contained in the deduced sequence of p64H1.

3.3.5 Preparation of a probe derived from 5'-flanking sequence isolated using circle- or concatamer-mediated RACE-PCR

A novel technique for the isolation of 5'-flanking regions of cDNA from regions of unknown sequence was described in 1995; circle- or concatamer-mediated RACE-PCR (cRACE; Maruyama *et al.*, 1995). This technique has several attractive features; it is rapid, inexpensive and simple. There is no requirement for PCR using universal primers as all the oligonucleotides used in the reaction are gene-specific. The sequence information obtained after cRACE is not a true representation of the sequence of the mRNA under study, rather it is an artifactual sequence generated by circularising cDNA. Using this procedure, however, sequence data may be obtained for regions of uncharacterised 5'-flanking cDNA. This technique was applied to the problem of isolating sequences 5'- to the clone *BS2*, and is summarised in Figure

a.



b.

1. P4445: 5'-CGGTGGTGACACTGAACACGACTCC3'

2. M7587: 5'-CGGTTGACCTGAAAAGG-3'

3. P3724: 5'-TCAACGGTGGTGACACTGAAC-3'

Figure 3.6 cRACE. a. Diagrammatic representation of cRACE. **b.** The sequences of the primers used for cDNA synthesis and amplification.

3.6, a. The sequences of the oligonucleotide primers used in first-strand cDNA synthesis and PCR amplification from the circularised template are shown in Figure 3.6, b. The first-strand cDNA synthesis reaction was set-up as follows;

rat brain poly(A)$^+$ RNA (1 µg/µl)	1 µl
cDNA synthesis primer (10 pmoles/µl)	1 µl
DEPC-treated deionized water	9 µl

This mixture was incubated at 70°C for 15 mins to denature the mRNA secondary structure, then placed on ice. The following were added;

5 X buffer (250 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 15 mM $MgCl_2$: Gibco BRL)	4 µl
0.1 M DTT	2 µl
dNTP mix (10mM each dNTP)	1 µl
[α^{32}P]-dCTP (3000 Ci/mmol)	1 µl

The reaction mixture was incubated at 39°C for 2 mins before the addition of 1 µl of reverse transcriptase (Superscript II, 2 U/µl). The reaction was incubated at 39°C for 1 hour before reaction termination, and mRNA template hydrolysis by the addition of 10 µl of 1 *N* NaOH, and further incubation at 39°C for 10 mins. The newly-synthesized first-strand cDNA was ethanol precipitated and resuspended in 12.5 µl of ligation buffer (25% (v/v) PEG 8000, 1 mM hexamine cobalt (III) chloride, 0.01 mM ATP, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 µg/µl BSA). The successful synthesis of first-strand cDNA directed from a *BS2*-specific oligonucleotide primer was confirmed by the electrophoresis of an [α^{32}P]-dCTP labelled aliquot (2.5 µl) of cDNA in a 6% denaturing polyacrylamide gel followed by

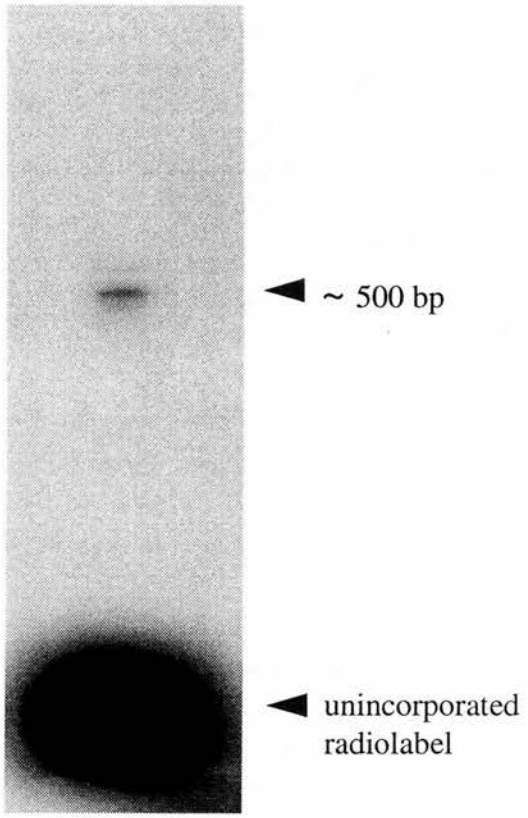


Figure 3.7 First-strand cDNA directed from primer P4445.

An aliquot of first-strand cDNA radiolabelled with [α^{32}P]-dCTP was electrophoresed in a 6% (w/v) denaturing polyacrylamide-urea gel. The autoradiogram revealed the presence of a cDNA of approximately 500 nucleotides. This cDNA was used subsequently in a cRACE amplification.

autoradiography (Figure 3.7). The autoradiogram of this gel revealed a single cDNA species of approximately 500 nucleotides. This cDNA was self-ligated in the ligation buffer by the addition of 1 µl (10 U) of T4 RNA ligase, and incubation at 22°C for 16 hours. Two microlitres of this ligation reaction were added to the following LDPCR mix;

10 X reaction buffer (Boehringer)	5 µl
dNTPs (10 mM each dNTP)	1.7 µl
primer 2 (10 pmoles/µl)	1 µl
primer 3 (10 pmoles/µl)	1 µl
sterile deionized water	38.6 µl

The thermal cycling conditions used were; 95°C, 1 minute, after which time 0.7 µl of *Taq /Pwo* DNA polymerase cocktail were added, followed by 30 cycles of 94°C, 10 seconds; 50°C, 10 seconds; 68°C, 90 seconds. The PCR product obtained from cRACE-PCR was approximately 510 bp in length (Figure 3.8): not long enough to extend all the way to the 5'-end of the complementary mRNA transcript, but long enough to extend into previously uncharacterised 5'-flanking regions of the gene. This PCR product was cloned into the vector pTAg, and sequenced from the T7 and SP6 phage promotor regions flanking the cloning site. Sequence analysis revealed that this clone (cRI) extended 5'-of the original clone *BS2*, commencing at relative nucleotide position 757 of bovine *p64*. This cDNA was used as a probe in the screening of a second cDNA library, constructed in the bacteriophage vector λgt 10.

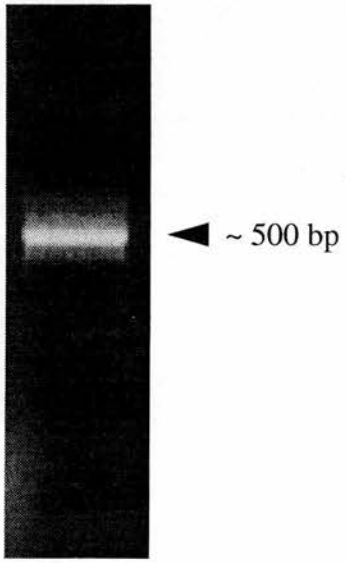


Figure 3.8 cRACE-PCR products (*cRI*). An aliquot (5 µl) of a cRACE-PCR was electrophoresed in a 1% (w/v) agarose gel. The product seen (cRI) was approximately 500 bp long, and was cloned into pTAg before sequencing.

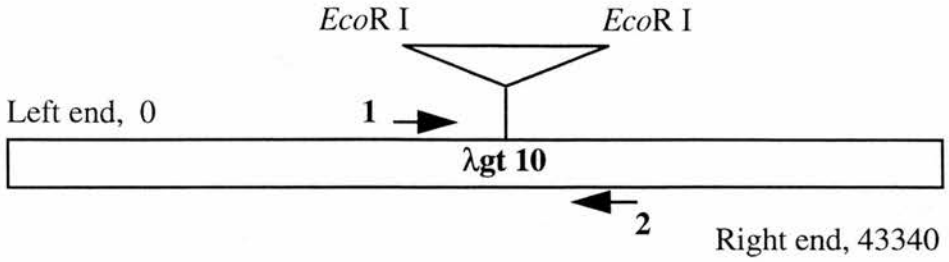
3.3.6 Screening a second, random-primed, cDNA library with cRI

The second cDNA library to be screened was constructed in a different manner to the original library constructed in λZAP II. The latter contained cDNA inserts synthesised from an oligo-d(T) cDNA synthesis primer, whereas the λgt 10 library contained a mixture of inserts synthesised in this manner and a proportion synthesised using random hexadeoxynucleotide primers. This library (5'-STRETCH, Clontech) was therefore likely to contain a proportion of clones representing cDNA complementary to regions of mRNA near the 5'- ends of transcripts. Approximately 1.2 x 10^6 independent clones were screened at low stringency using an [α^{32}P]-dCTP-labelled probed derived from cRI. The first round of screening resulted in 12 plaques being isolated and chosen for further analysis. A secondary screen in the same manner revealed 5 plaques to be true positives, which were isolated and their recombinant phage particles allowed to diffuse into 1 ml of SM buffer containing 20 µl of chloroform. These samples were used as templates for the PCR-amplification of phage-vector inserts using oligonucleotide primers against sequences flanking the vector cloning site.

3.3.7 Amplification and cloning of λgt 10 inserts

Unlike λZAP II, the phage vector λgt 10 does not possess helper phage excision recognition sequences for the *in vivo* excision of phagemid vectors containing cDNA inserts. In order to sub-clone the cDNA inserts from λgt 10 into a plasmid vector, the inserts were amplified using PCR with two oligonucleotide primers flanking the *Eco*R I cloning site of λgt 10. An aliquot (1 µl) of each purified plaque sample was

a.



1. *5'-screening primer*

5'-AGCAAGTTCAGCCTGGTTAAGT-3'

2. *3'-screening primer*

5'-TTATGAGTATTTCTTCCAGGG-3'

b.

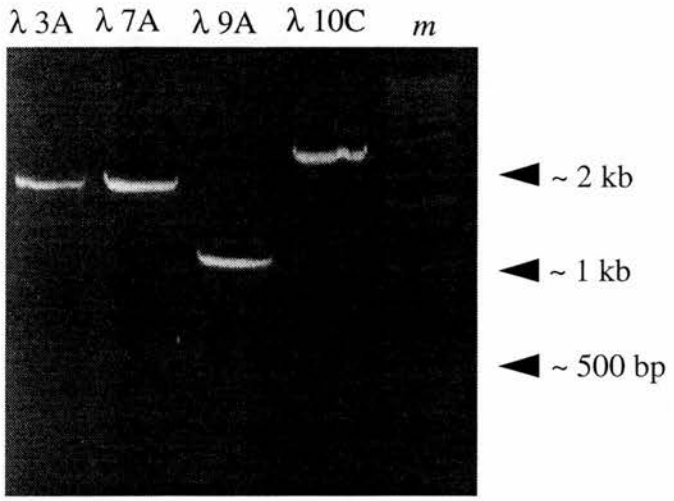


Figure 3.9 Sub-cloning inserts derived from λgt 10 phage vectors.

a. Relative positions and sequences of λgt 10 screening primers. The cloning site (*Eco*R I) is at position 32710, relative to position 0 at the left arm. **b.** Aliquots (5 µl) of cDNA inserts amplified using these primers were electrophoresed in a 1% (w/v) agarose gel.

frozen quickly in liquid nitrogen, and allowed to thaw at room temperature. The treated aliquots were then used directly in a Long-Distance PCR (LDPCR) using a polymerase cocktail of *Taq* polymerase and *Pwo* polymerase (thermal cycling conditions; 94°C, 10 seconds; 50°C, 10 seconds; 68°C, 2 mins, 30 cycles). The oligonucleotide primers used are presented in Figure 3.9, a., and an agarose gel showing the amplified PCR products in Figure 3.9, b. LDPCR was used in this experiment as the insert sizes within the phage vectors were potentially very large, and the polymerase cocktail is less prone to introduce polymerase errors than *Taq* alone (Barnes, 1994; Cheng *et al.*, 1994 a, b). The PCR products were then cloned into pTAg, and sequenced from the T7 and SP6 phage promotor sites.

3.3.8 Sequence analysis

Sequencing of the 5 cDNA clones described above revealed that 2 of the clones, λ 8 and λ 9A, contained a consensus sequence identical to the Kozak sequence for translational initiation (GGCCATGG; Kozak, 1984 a, b and c). The clone λ 9A was renamed *p64H1* (*p64 Homologue 1*). This sequence was preceded by 165 bp of 5'-UTR, the deduced amino acid sequence of which contained termination codons in each reading frame (Figure 3.10). The existence of additional 5'-UTR within *p64H1* mRNA cannot be ruled out. The deduced amino acid sequence following the putative initiation codon contained an open reading frame of 762 bp, predicted to encode a protein of 28.64 kDa. Optimal alignment of this sequence with that of bovine p64 revealed p64H1 to be 71% identical with the *C*-terminal half of p64 (Figure 3.11). A Kyte-Doolittle hydropathy plot (Kyte and Doolittle, 1982) using the

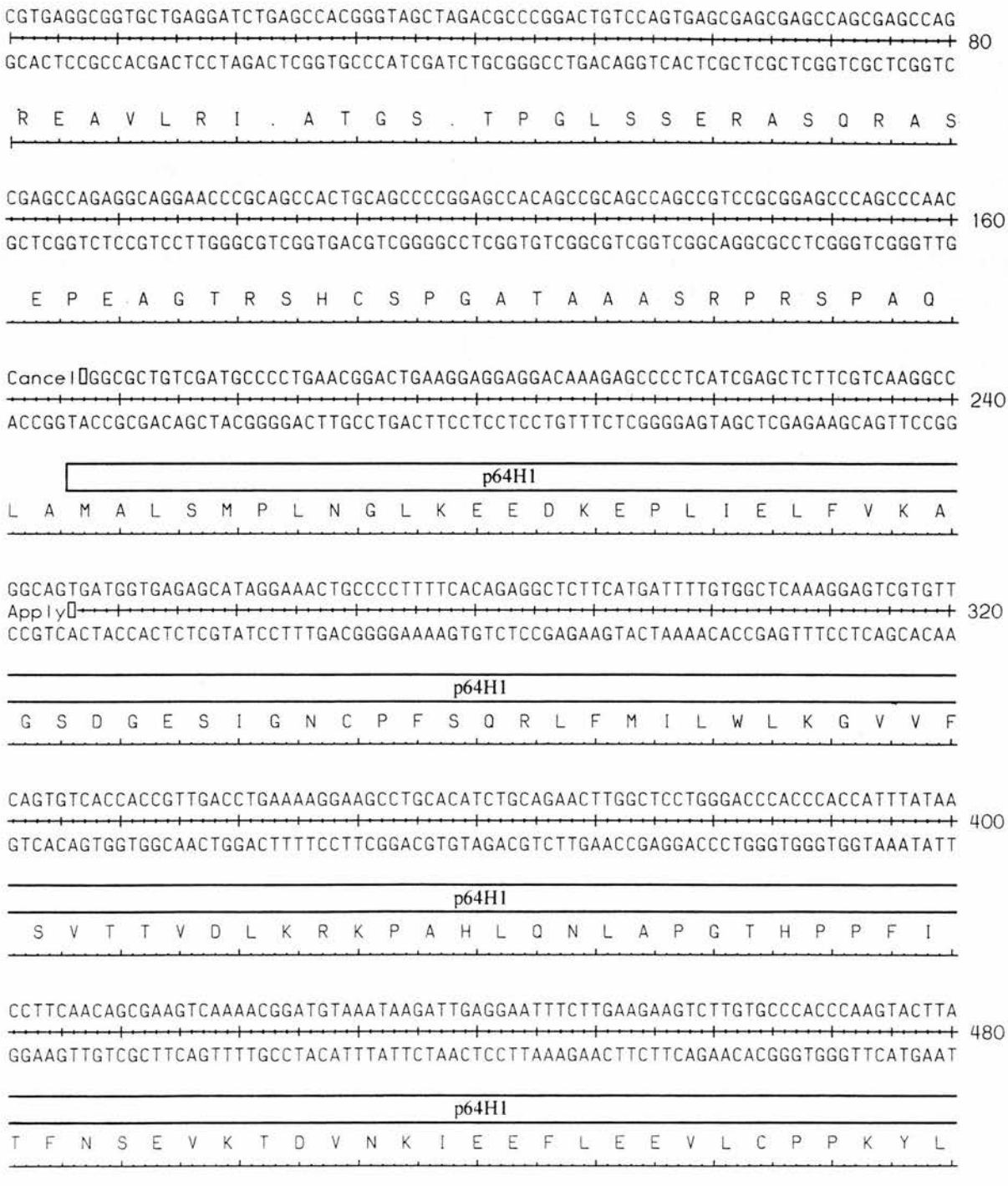


Figure 3.10 The nucleotide and deduced amino acid sequence of p64H1

(continued overleaf).

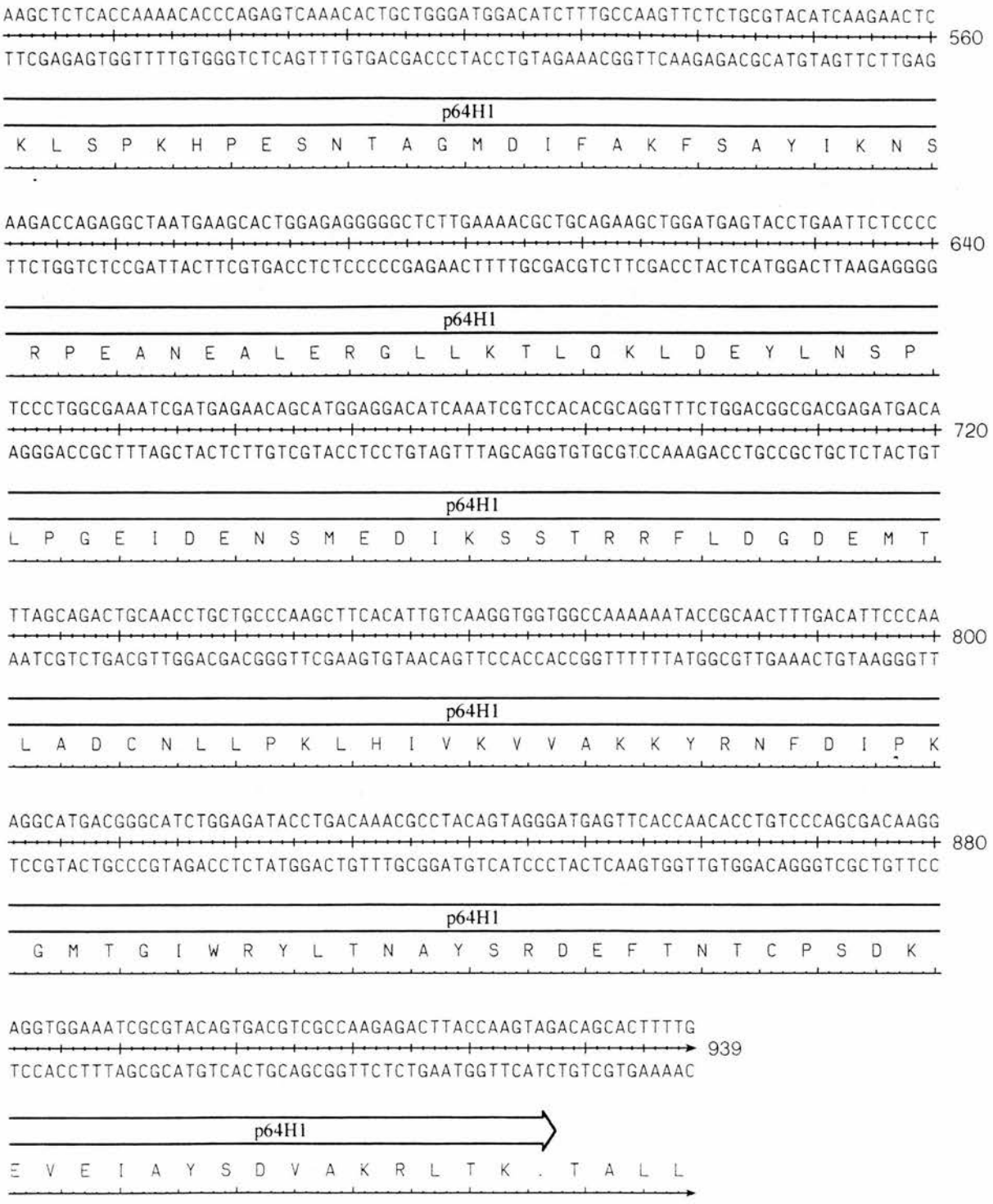


Figure 3.10 The nucleotide and deduced amino acid sequence of p64H1

(continued from overleaf).

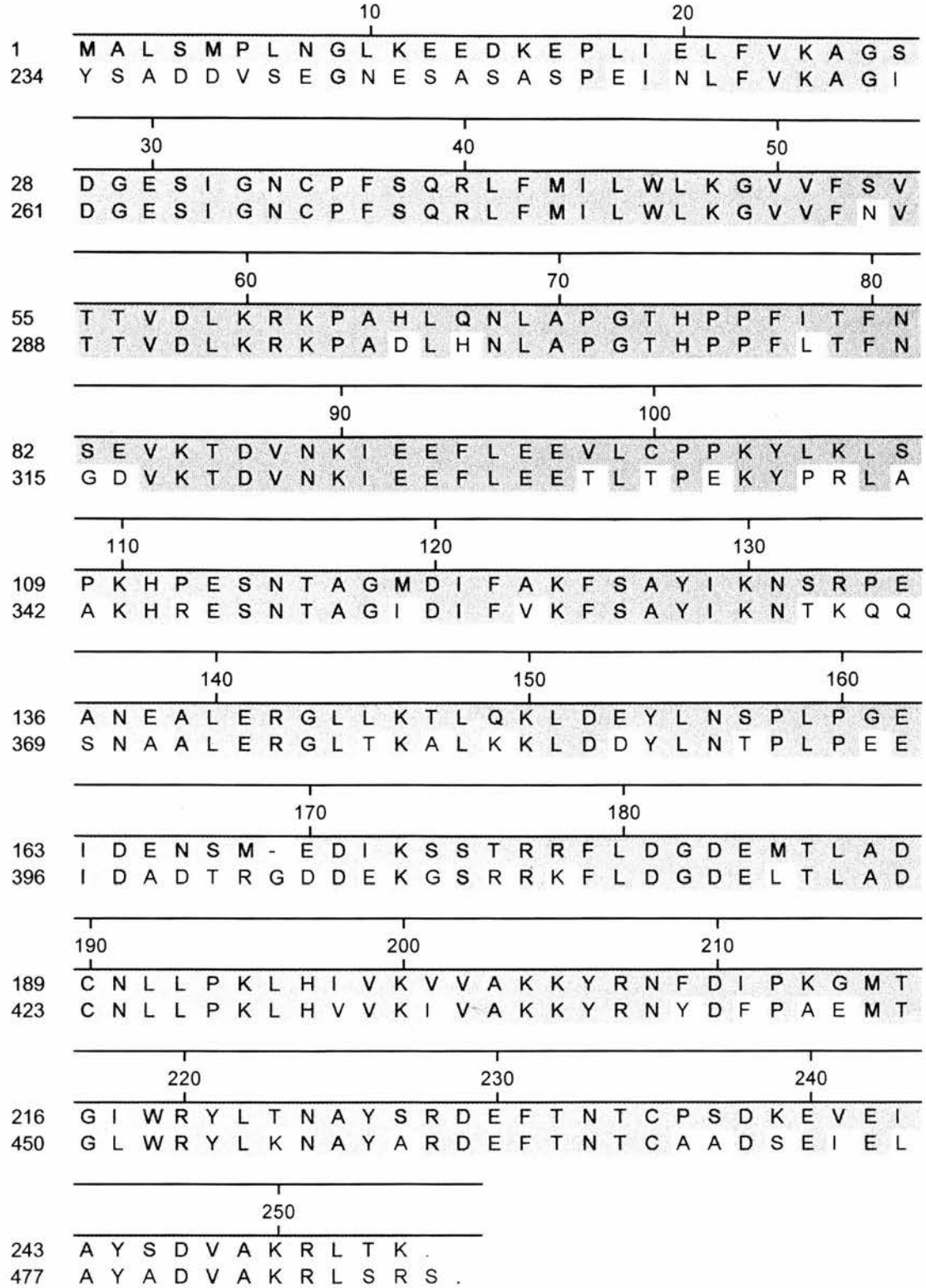


Figure 3.11 An optimal alignment between the deduced amino acid sequences of p64H1 (top) and p64 (lower sequence). Alignments were performed using the Clustal algorithm within the MegAlign program of the DNAStar package. Residues matching p64H1 are shaded in yellow. The identity is 71%.

deduced amino acid sequence of p64H1 predicted a region of 18 amino acids (residues 39-56) capable of forming a transmembrane (Tm) domain. The potential Tm domain was near the *N*-terminus of p64H1, with a larger (~ 25 kDa), *C*-terminal domain predicted to reside within the cytoplasm. The *C*-terminal domain contained four consensus recognition sites ([S/T]X[R/K]; Kishimoto *et al.*, 1985) for protein phosphorylation by protein kinase C (PKC; Ser 107, Ser 173, Thr 174 and Ser 235) and a single consensus site for phosphorylation by protein kinase A (Thr 252). In addition to these sites, a consensus PKC phosphorylation site was predicted at the beginning of the putative Tm domain (Ser 37).

3.4 Summary

A clone was isolated, using a combination of RTPCR, conventional cDNA library screening and cRACE, which encoded the complete ORF of a homologue of bovine *p64*, *p64H1*. This clone contained ~ 165 bp of 5'-UTR and ~ 500 bp of 3'-UTR. Sequence analysis showed that *p64H1* was ~70% identical to bovine *p64*, where the sequences overlapped. *p64H1* was predicted to encode a protein approximately half the size of bovine p64 (of ~ 30 kDa), corresponding to the *C*-terminal half of the latter. The deduced amino acid sequence of p64H1 was predicted to contain a single Tm domain. The putative *C*-terminal domain contained multiple consensus sites for protein phosphorylation, suggesting a cytoplasmic location. No consensus sites for glycosylation were found.

Chapter 4

Localisation of p64H1

4.1 Introduction

Determination of the cellular and intracellular localisation of a mRNA or protein can provide clues to its function. A thorough characterisation at every level was undertaken for p64H1: the tissue distribution of *p64H1* mRNA was determined using Northern hybridisation, followed by further localisation within rat brain using mRNA *in situ* hybridisation. Experiments were also performed to localise the *p64H1* gene product by detecting expression in tissues using Western blotting and immunohistochemistry. Finally, the *intracellular* localisation of heterologously-expressed p64H1 in cultured cells was studied using indirect immunofluorescence.

4.2 Determination of *p64H1* transcript size and tissue distribution using Northern hybridisation

Total RNA was prepared from rat brain, poly(A)$^+$ mRNA prepared from these samples, and 2 µg electrophoresed in a 1% (w/v) denaturing agarose-formaldehyde gel. These samples were transferred to a nylon membrane, and probed with a 346 bp *Eco*R I restriction fragment of *BS2,* labelled with [α^{32}P]-dCTP. The blot was washed at high stringency (final wash: 0.2 X SSC, 65°C, 20 mins). In addition, a commercially prepared Northern blot (MTN™, Clontech) containing mRNA from brain, cerebellum, heart, kidney, spleen, lung, testis and liver was probed in a similar manner. Autoradiograms obtained from these Northern hybridisations are presented in Figure 4.1. The full-length transcript encoding p64H1 was found to be approximately 4.3 kb in length, and widely-expressed. This transcript was found to be abundant in brain, liver , testis and lung, but expressed at lower levels in the other tissues studied. When the Northern blots were probed at a reduced, moderate

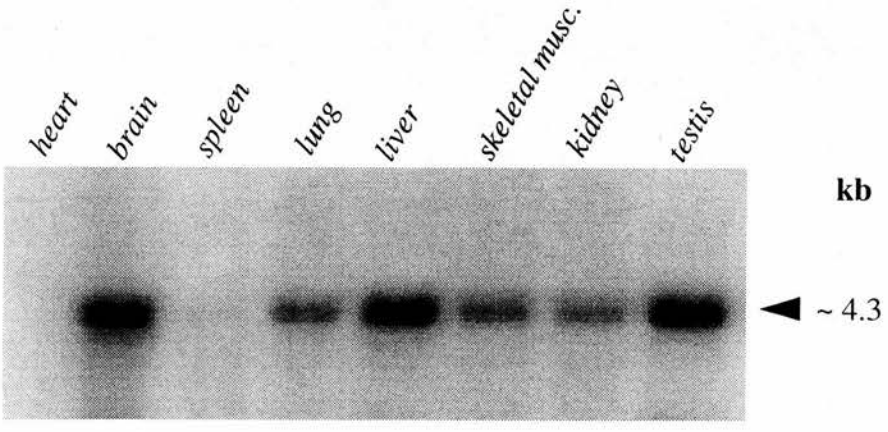


Figure 4.1 Northern blot washed at high stringency.

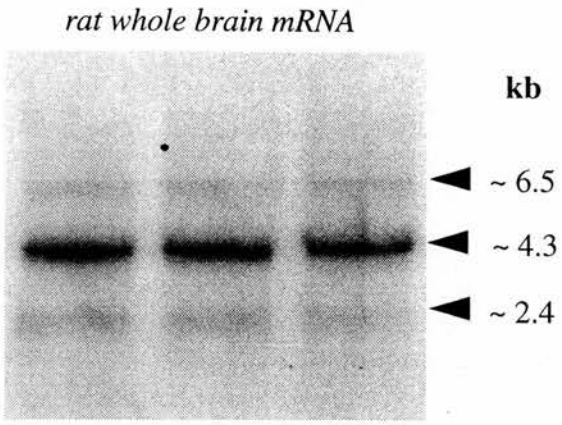
Northern blot of rat tissue mRNA probed with a 346 bp *Eco*R I restriction fragment of *BS2* labelled with [α^{32}P]-dCTP, and washed at high stringency. A single transcript of approximately 4.3 kb was revealed in most tissues.

stringency (final wash: 2 X SSC, 0.1% (w/v) SDS, 65°C, 30 mins.), three mRNA species hybridised with the probe, of 2.4 kb, 4.3 kb and 6.5 kb. The hybridisation signals obtained for the 2.4 kb and 6.5 kb species were reduced compared to the signal for the 4.3 kb transcripts (Figure 4.2). These additional transcripts may represent alternatively spliced forms of *p64H1*, or other members of a gene family. The full-length mRNA encoding bovine p64 was previously reported to be 6.5 kb long (Landry *et al.*, 1993), raising the possibility that the *BS2* probe had cross-hybridised with rat brain p64. The expression of *p64H1* was found to be developmentally regulated. Northern blot analysis of embryonic mRNA performed as before and washed at moderate stringency demonstrated that *p64H1* is expressed only in late embryonic development. No *p64H1* could be detected at day (d) 16 of development, with reduced expression (relative to adult levels) detectable at d 20 of embryonic development (Figure 4.3).

4.3 *In situ* hybridisation

The localisation of *p64H1* mRNA was further studied in rat brain using mRNA *in situ* hybridisation. A 45-mer antisense oligonucleotide (5'CCTTTTCAGGTCAACGGTGGTGACACTGAACACGACTCCTTTGAG-3') was synthesised, and 5'-end labelled with [α^{35}S]-dATP using terminal deoxynucleotidyl transferase. A complementary, sense oligonucleotide was synthesised and labelled in the same manner for use as a negative control. These labelled oligonucleotides were used to probe rat brain sections, followed by autoradiography. The autoradiograms showed intense hybridisation signals in the

a.



b.

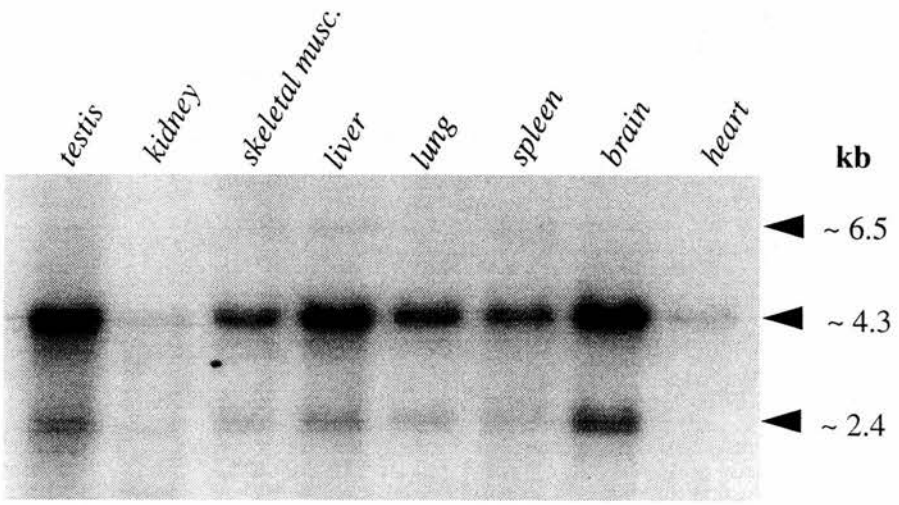


Figure 4.2 Northern blot washed at moderate stringency.

a. Rat brain mRNA probed with a *BS2*-specific probes as before, but washed to a moderate stringency. Three transcripts hybridised with the probe, of 6.5 kb, 4.3 kb and 2.4 kb. **b.** Northern blot as in **a.**, probing mRNA extracted from a variety of rat tissues (MTN, Clontech).

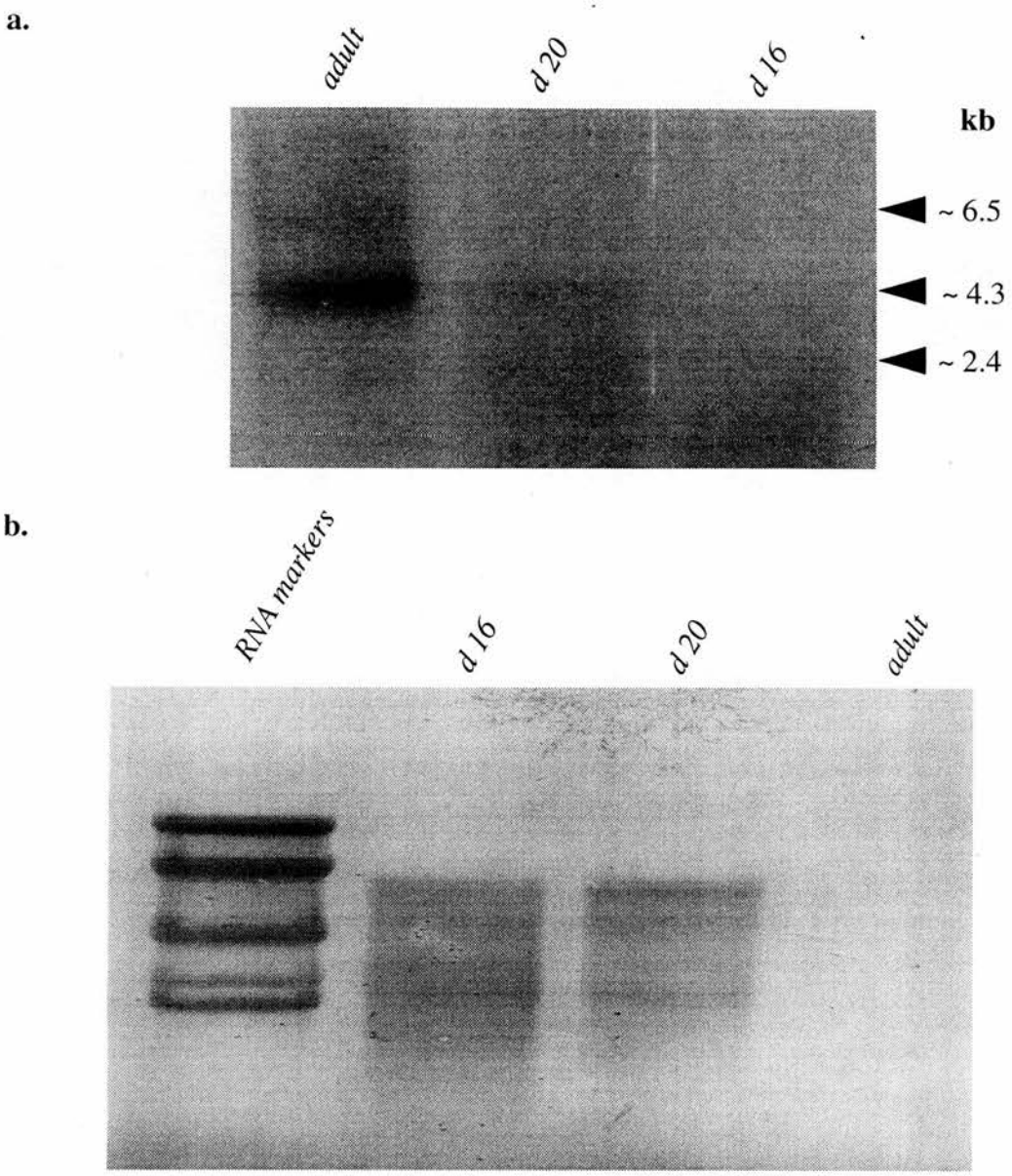
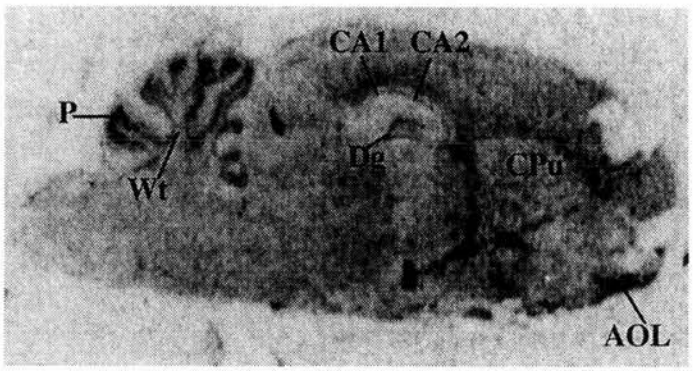


Figure 4.3 Northern blot demonstrating the developmental expression of p64H1. a. Rat brain mRNA extracted from adult, d 20 and d 16 embryos probed with a *BS2*-specific probe and washed to a moderate stringency. **b.** The membrane was stripped of probe, and stained with methylene-blue to examine the relative transfer of samples.

a.



b.

Figure 4.4 mRNA *in situ* hybridisation localisation of *p64H1* in rat brain sections. a. mRNA *in situ* hybridisation using a 45-mer oligonucleotide to probe rat brain sections. Hybridisation signal was seen in the cerebellum, hippocampus and dentate gyrus. **P**, cerebellar Purkinje cell layer; **Wt**, cerebellar white matter; **CA1**, **CA2**, areas of Ammon's horn; **Dg**, dentate gyrus; **AOL**, anterior olfactory lateral nucleus; **CPu**, caudate putamen. **b.** mRNA *in situ* hybridisation performed as before using a complementary, sense probe as a negative control. No specific staining was seen. The sections were autoradiographed for 12 d.

cerebellum and hippocampal areas of rat brain (Figure 4.4, a), with moderate hybridisation in the cortex. No specific staining was observed when the sense probe was used (Figure 4.4, b).

4.4 Western blotting

4.4.1 Raising of an anti-p64H1 antiserum and Western blotting

The 346 bp *Eco*R I restriction fragment of *BS2* was ligated into the expression vector pAX11 (Zueco and Boyd, 1992), to create an "in-frame" fusion with the sequence encoding *Staphylococcal* Protein A. After transformation of *E. coli* strain XL1-Blue, clones containing the recombinant plasmid were isolated. The integrity of the cDNA insert was confirmed by sequencing, and the ligation was confirmed to be "in-frame". A single transformed colony was innoculated into 100 ml of LB-kanamycin media, and grown until the absorbance of the culture at OD_{600} reached 0.6. At this point, expression from the *Lac* promotor was induced by the addition of IPTG to the media, at a final concentration of 100 mM. The expression of a unique protein of the predicted size (~ 36 kDa), inducible with IPTG could be detected 2, 3 and 4 h after induction by SDS-PAGE followed by Coomassie Brilliant Blue staining. Further analysis of pellet and supernatent fractions of lysozyme-lysed, sonicated bacteria revealed the recombinant protein to be almost completely insoluble at 37°C. The insolubility permitted the partial purification of recombinant protein by the isolation of "inclusion bodies", (Marston *et al.*, 1984; Sambrook *et al.*, 1989; pp 17.39-17.40). One hundred millilitres of cells induced to express the fusion protein for 4 hours as described above were collected by centrifugation at

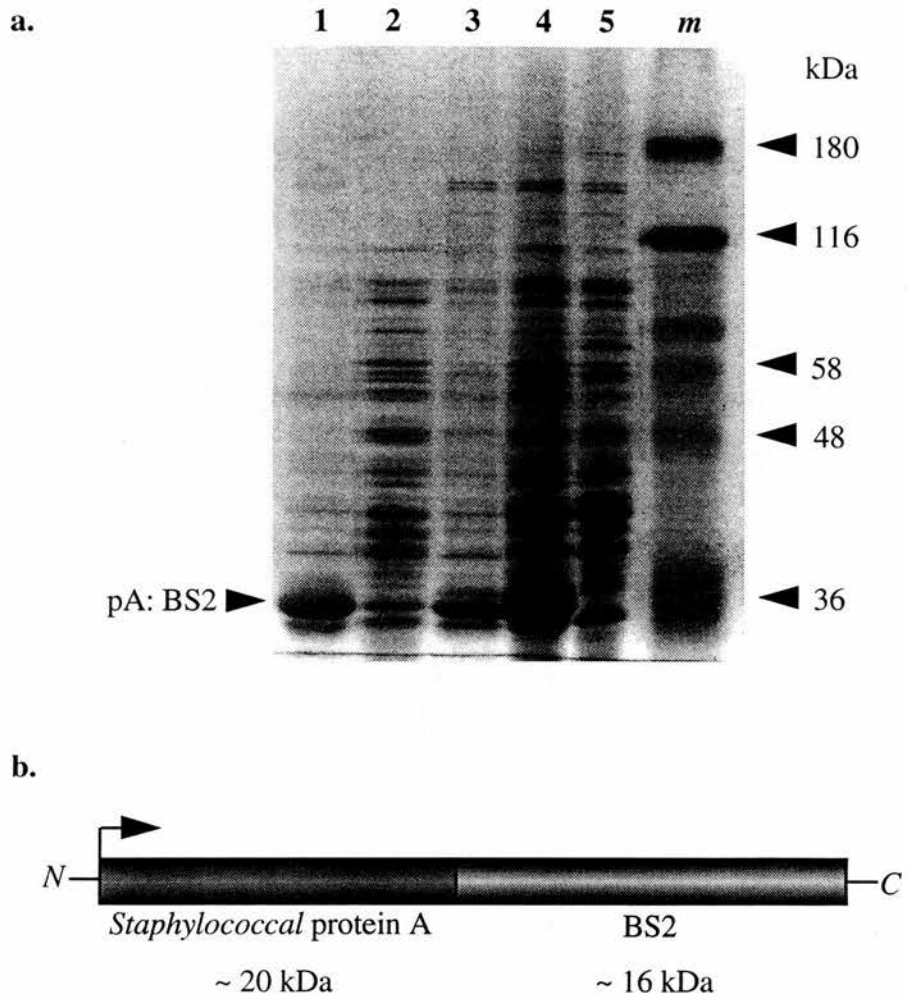


Figure 4.5 Partial purification of a protein A:BS2 fusion protein.

a. Expression and partial purification of protein A:BS2. Lane 1: partially purified inclusion bodies, Lane 2: supernatent fraction of lysed cells, Lane 3: pellet fraction of lysed cells, Lane 4: IPTG-induced total cell lysate, 4 h post-induction, Lane 5: uninduced total cell lysate. *m:* Sigma molecular weight markers. The samples were electrophoresed in a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. **b.** Diagrammatic representation of the protein A:BS2 fusion protein.

4500 x *g* for 15 mins at 4°C. The cells were resuspended in 10 volumes of ice-cold lysis buffer (150 mM NaCl, 10 mM EDTA, 100 mM Tris-HCl, pH 8.5), containing protease inhibitors (Complete Inhibitor tablets, Boehringer) and lysozyme added to a final concentration of 100 µg/ml. The cell suspension was incubated at 37°C for 15 mins, followed by 2 x 30 s sonication pulses at high power. The lysed cells were pelleted, and the supernatent and pellet fractions retained and subjected to SDS-PAGE (Figure 4.5). The partially-purified recombinant protein shown in Figure 4.5 was used to immunise rabbits as described in Chapter 2. After four injections, antisera (Ab990) were obtained which reacted with a protein of apparent molecular weight of 43 kDa in a variety of rat tissues. Immunoblot analysis demonstrated the 43 kDa protein to be present in rat brain, heart, kidney and liver (Figure 4.6, a). Further analysis, based upon the observation that *p64H1* mRNA is expressed in high levels in rat cerebellum (Figure 4.3, a) revealed that this protein species is present in higher levels in the cerebellum than in the forebrain (Figure 4.6, b). Further characterisation, immunoblotting differentially-centrifuged samples from rat whole brain, demonstrated that the immunoreactive protein was present in the P3 microsomal pellet fraction, with lower levels in the P1 fraction (Figure 4.7). No protein was detected in the P2 or supernatent fractions (Howell *et al.*, 1996).

4.5 Immunohistochemistry

To further localise p64H1 protein to specific brain regions, rat brain sections were subjected to immunohistochemical studies. Intense staining was observed in the rat hippocampal CA1-CA3 regions, the dentate gyrus, but especially in the Purkinje cell layer of the cerebellum (Figure 4.8, a).

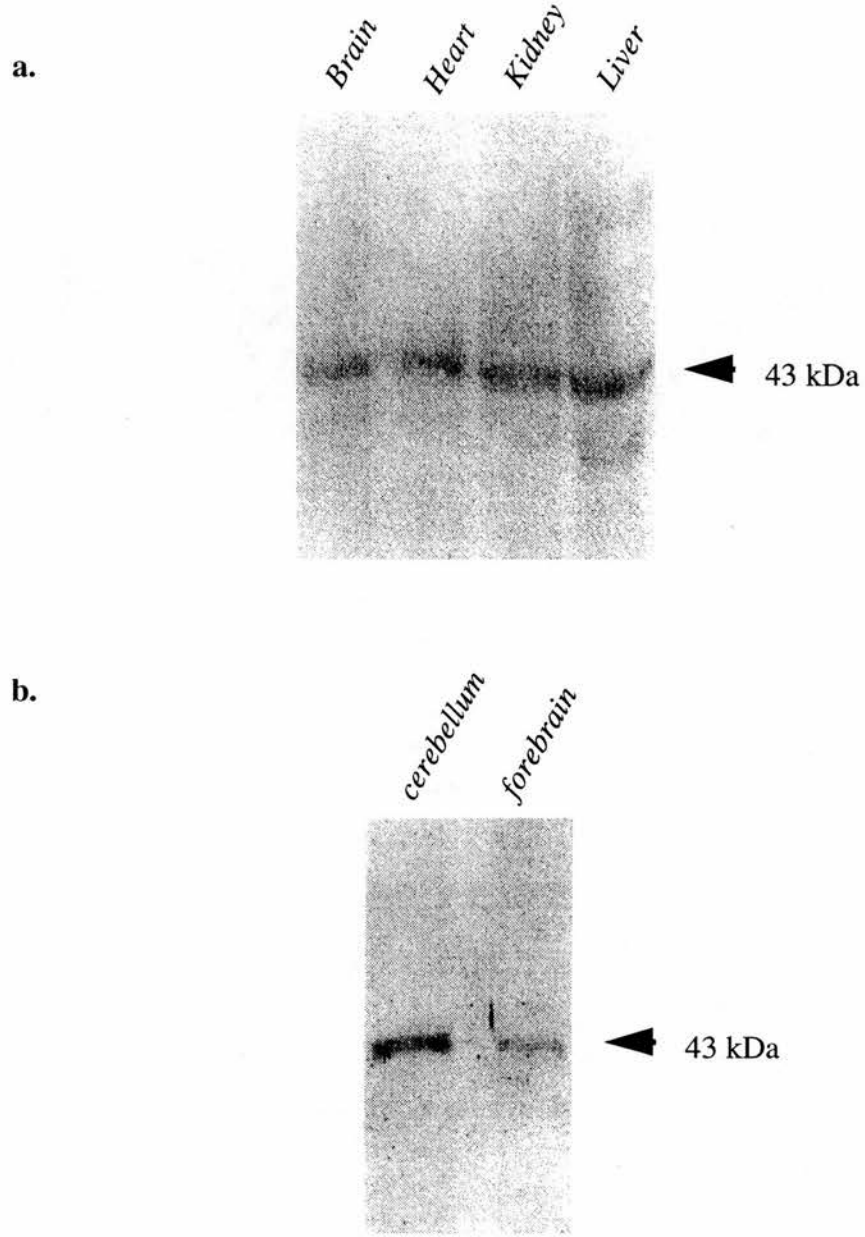


Figure 4.6 Immunoblot analysis using Ab990. a. Western blot analysis of rat tissue protein extracts. Ab990 recognised a 43 kDa protein in brain, heart, kidney and liver. **b.** The 43 kDa protein was more abundant in the cerebellum than the forebrain. Approximately 10 μg of each protein extract were blotted, and reactive proteins revealed using an alkaline-phosphatase conjugated second antibody and NBT/BCIP.

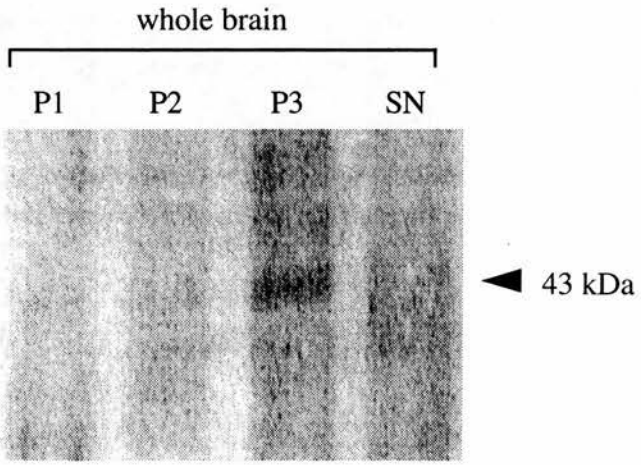


Figure 4.7 Localisation of native p64H1 to the P3 microsomal fraction of rat brain homogenate. Immunoblot analysis of differentially-centrifuged membrane fractions from rat brain homogenate. Antiserum 990 recognised a 43 kDa protein abundant in the P3 fraction. Ten micrograms of protein were loaded in each lane, and reactive proteins revealed using an alkaline-phosphatase conjugated second antibody and NBT/BCIP. SN: supernatent fraction.

No specific staining was observed when pre-immune serum was used in place of the primary antiserum (Figure 4.8, b).

4.6 Indirect immunofluorescence

The data obtained for p64H1 protein and mRNA localisation suggested that p64H1 was an intracellular protein of 43 kDa expressed in the rat brain, notably in Purkinje cells and cells of the hippocampus. Further work was undertaken to localise the protein to an intracellular compartment. A *Kpn* I-*Xba* I restriction fragment was sub-cloned from the plasmid pTAg. p64H1 into the mammalian expression vector pCI. This restriction fragment contained the entire ORF encoding p64H1, 165 bp of 5'-UTR and approximately 500 bp of 3'UTR. This plasmid construct was used to transfect HEK293 cells using Tfx-20 reagent, and indirect immunofluorescence was used to detect recombinant protein. Expression of *p64H1* could be detected 48 h post-transfection (Figure 4.9). No p64H1 could be detected in cells transfected with pCI alone, and no specific immunofluorescence was seen when pre-immune serum from rabbit 990 was used as the primary antibody. The staining observed when Ab990 was used was restricted to a perinuclear, membranous structure reminiscent of endoplasmic reticulum. The nuclear membrane could be clearly visualised in some cells. The position of the nucleus was confirmed by staining for cellular DNA using Hoechst dye number 33258. In order to determine whether expressed p64H1 protein was being correctly targeted in HEK293 cells, or whether it was being inappropriately processed by this epithelial cell-line, cells of the cell-line HT4 was transfected in the same manner. HT4 cells are neuronal in origin (Frederiksen *et al.*,

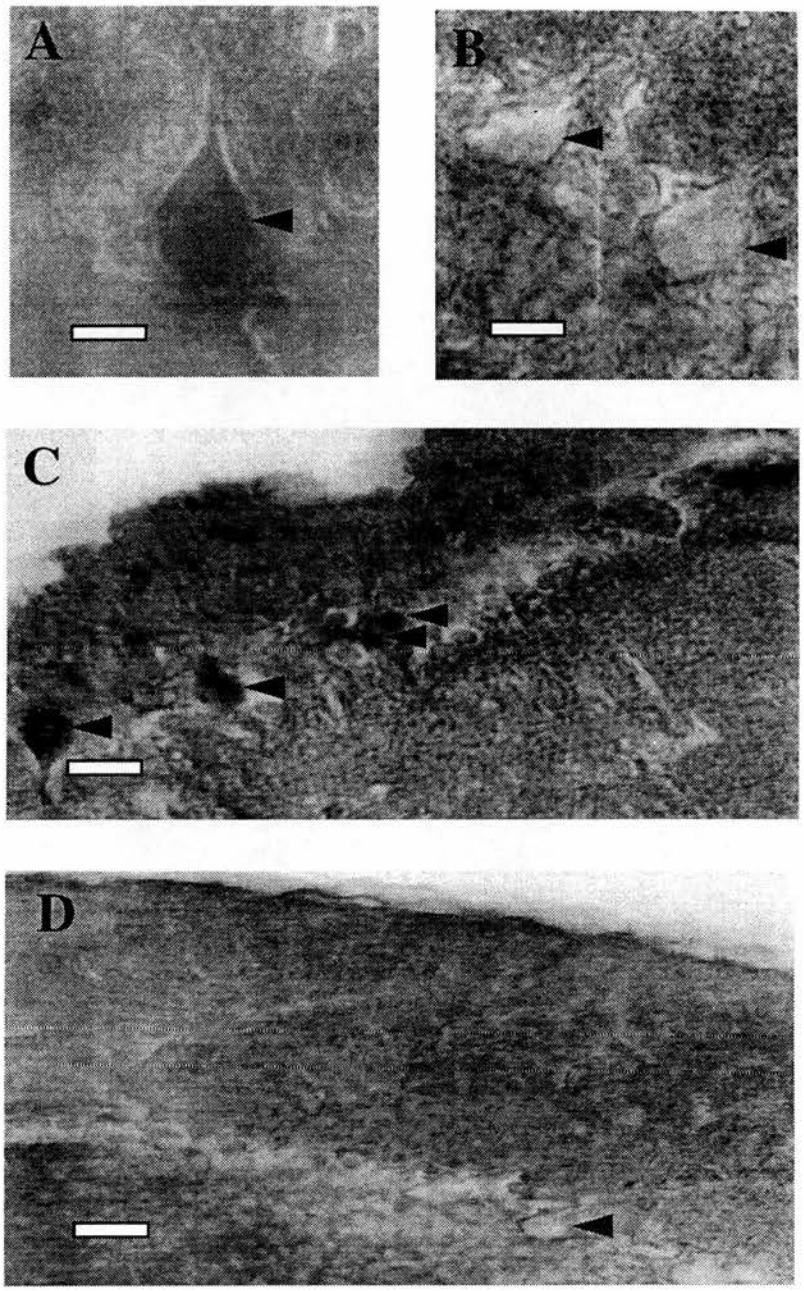


Figure 4.8 Immunohistochemical localisation of p64H1 within rat brain sections using Ab990. High power magnification (1000 X) of a 15 micron rat cerebellum section stained with Ab990 (Panel A). The cell bodies and processes of Purkinje cells were intensely stained (arrowheads). Corresponding sections treated with pre-immune serum showed no staining of Purkinje cells (Panel B). Panel C: low power (200 X) Ab990-stained section. Panel D: pre-immune treatment as before (200 X). Scale bars: ~ 10 microns (Panels A and B), ~ 50 microns (Panels C and D). Reactive proteins were detected using a biotin-conjugated second antibody, and streptavidin-HRP. Immune complexes were stained using AEC/peroxide (Sigma ExtraAvidin staining kit).

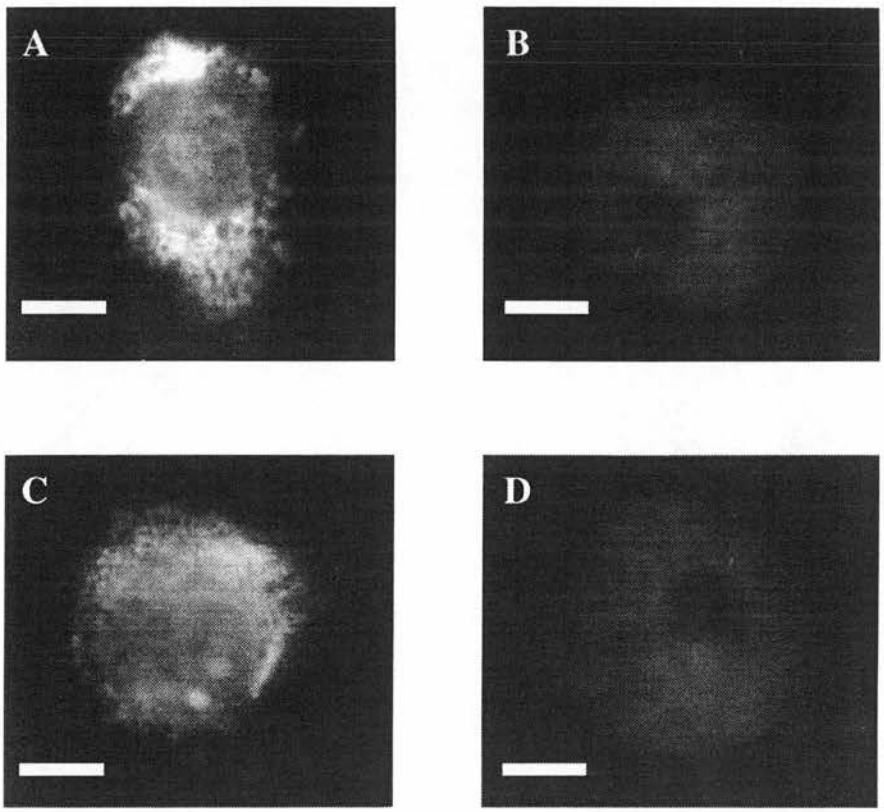


Figure 4.9 Indirect immunofluorescent detection of p64H1 expression in HEK293 cells. The expression of p64H1 was restricted to a perinuclear vesicular localisation (Panels A and C). Hoechst 33258 stained nuclei are shown in Panels B and D. (Scale bars: approximately 1 micron).

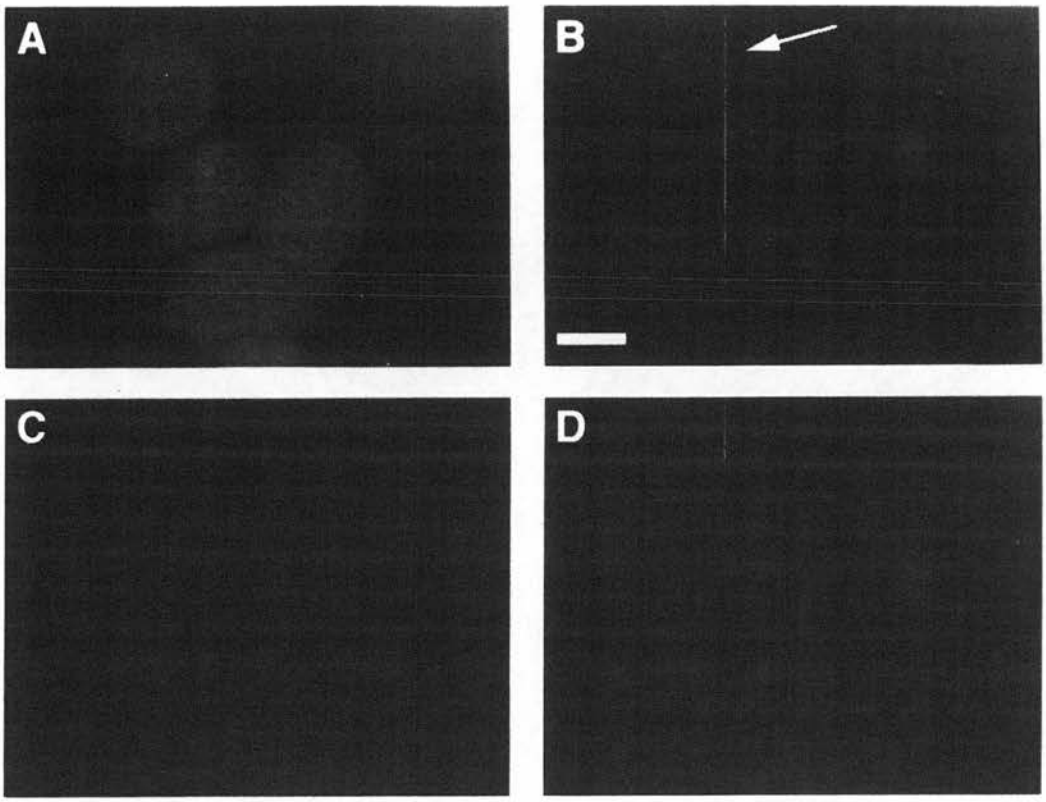


Figure 4.10 Indirect immunofluorescent detection of p64H1 expressed in HT4 cells. Panel A: Transfected cells were stained for cellular DNA using Hoechst dye number 33258; Panel B: the cells were stained using Di-O-C_5(3) to visualise the membranes of the ER; Panel C: p64H1 expression was detected using Ab990 and a goat anti-rabbit IgG rhodamine-conjugated second antibody; Panel D: superimposition of Panels B and C demonstrated the co-localisation of recombinant p64H1 and stained ER membranes. Scale bars: 2 μm. The samples were examined under oil at 400 X magnification, and images captured using a Hamamatsu C5810 chilled CCD camera and standard Nikon TRITC, FITC and UV filter cube. (The photographs were taken by Dr. R. Ribchester, Dept. of Physiology, University of Edinburgh.)

1988) and behave in a neuron-like manner (Frederikson *et al.*, 1988). The staining pattern observed in these experiments was similar to that observed when using HEK293 cells (Figure 4.10). The HT4 cells were also stained using Hoechst dye 33258, and in addition the short-chain cationic dicarbocyanine dye Di-O-$C_5(3)$ was used. These dyes have been demonstrated to partition with the membranes of reticular structures within the cell, shown to be ER (Terasaki *et al.*, 1984; Terasaki *et al.*, 1992). The pattern of staining observed when this dye was used was identical to that seen for p64H1 immunoreactivity, and superimposition of the images obtained from rhodamine- and FITC-wavelengths demonstrated that the p64H1 staining pattern overlapped exactly with the stained ER-membranes (Figure 4.10).

4.7 Summary

The cellular and sub-cellular distributions of p64H1 mRNA and protein were investigated using a variety of techniques. The mRNA transcript encoding p64H1 was demonstrated to be a widely-distributed transcript of ~ 4.3 kb. The protein product was shown to be a widely-expressed, 43 kDa species. Both the mRNA and protein were found to be abundant in the Purkinje cell layer of the cerebellum. Western blot analysis demonstrated p64H1 to be abundant in the P3 microsomal membrane fraction of rat tissue homogenates, with little protein detected in other fractions. The localisation of heterologously-expressed p64H1 in two cell-lines, HEK293 and HT4 cells, was found to be restricted to the ER.

Chapter 5

In vitro translation of

p64H1

5.1 Introduction

Cell-free protein translation systems provide an extremely useful tool for the characterisation of proteins encoded by specific cDNAs. The primary advantage of *in vitro* translation systems over *in vivo* protein expression is the ability to "program" a cell-free system with a single cDNA or mRNA, and visualise the protein product specifically by the inclusion of a radiolabelled amino acid, followed by SDS-PAGE and autoradiography. Several cell-free translation systems have been developed, using both eukaryotic and prokaryotic cell lysates. The format of choice throughout this study was a Rabbit Reticulocyte Lysate coupled transcription and translation system (TnT) supplied by Promega.

The rabbit reticulocyte lysate protocol was originally developed by Pelham and Jackson (1976) and requires the addition of a mRNA transcript to direct translation *in vitro*. Endogenous cellular mRNA is destroyed by the addition of micrococcal nuclease, which is completely dependent upon Ca^{2+} for nuclease activity. After nuclease treatment, the reticulocyte lysate is treated with EDTA to chelate the Ca^{2+} ions, rendering the micrococcal nuclease inactive. Commonly, the transcript used in such systems is synthesised *in vitro* (Kreig and Melton, 1984) from a linerarized plasmid template containing the cloned gene of interest downstream of a phage promotor. A coupled transcription/translation system obviates the requirement for a separate *in vitro* transcription step, allowing the addition of a cDNA to the cell lysate (Craig *et al.*, 1992). In such a system, all the required components for *in vitro* transcription directed by a cloned phage-promotor are included in the translation mix. As the monovalent and divalent ion requirements for transcription (Melton *et al.*,

1984) and translation (Jackson *et al.*, 1983) differ significantly, and the conditions in a coupled system are closer to those optimal for *in vitro* translation (Craig *et al.*, 1992), the transcription step of such a reaction remains relatively inefficient. However, sufficient RNA is produced to effectively direct *in vitro* translation, especially if the phage promotor used is powerful, as is the case for the T7 promotor. The TnT system format chosen required that the cDNA of interest be cloned downstream from the T7 promotor in a plasmid vector.

In addition to the ability to determine the ability of a particular cloned cDNA to direct protein synthesis, and to determine the molecular weight of the protein product, several cotranslational processes may be studied using an *in vitro* translation system. The inclusion of microsomal membranes in the translation mix permits protein translocation, signal peptide cleavage and core glycosylation (Blobel and Dobberstein, 1975; Yost *et al.*, 1983). Such modifications may often be detected by migration differences during SDS-PAGE. Furthermore, integral membrane proteins may be distinguished from secretory or peripheral membrane proteins by treating the microsomes at high pH, which will extract the vesicle contents as well as dissociate peripheral membrane proteins (Fujiki *et al.*, 1982; Gilmore and Blobel, 1985). Once a protein product has been demonstrated to be an integral membrane component, in some cases the membrane topology may be predicted using a combination of hydropathicity predictions and protease protection assays. If the product of *in vitro* translation is an integral membrane protein, then it will be partially translocated into the microsomal vesicle during translation. The addition of proteinase K to the reaction after this event allows protein domains

outside the vesicles to be degraded, whereas internal and transmembrane domains will be afforded protection from degradation (Feng *et al.*, 1995). The membrane topology of p64H1 was determined using a combination of predictive modelling and biochemical analyses. The membrane topologies of ion channels have been well studied (e. g. Tusnady *et al.*, 1997; Shih and Goldin, 1997; Machaca and Hartzell, 1997; Stanley and Manella, 1997; Therien *et al.*, 1997), but the model proposed for bovine p64 relied entirely on hydropathicity predictions (Landry *et al.*, 1993), and as such was unsatisfactory. It was proposed that bovine p64 possessed 2, or possibly 4 transmembrane regions, but this work has never been supported experimentally. The amino acid sequence deduced from the *p64H1* nucleotide sequence shows identity with the *C*-terminal half of p64, and the hydropathicity profiles over the homologous regions are extremely similar. Any information determined regarding the membrane topology of p64H1 may therefore be useful in commenting on the structure of the *C*-terminal half of p64.

5.2 *In vitro* expression of p64H1

A 50 µl coupled transcription/translation reaction was programmed with the plasmid vector pTAg.p64H1, in the presence or absence of canine pancreatic microsomal membranes, and aliquots were analysed by SDS-PAGE and autoradiography as described in Chapter 2. The protein product directed from pTAg.p64H1 had an apparent molecular weight of approximately 33 kDa (Figure 5.1). This was in agreement with the deduced protein based upon the ORF of *p64H1*, which had a predicted molecular weight of approximately 29 kDa. Translation in the presence of

microsomes did not alter the apparent weight of the protein product (Figure 5.1). The interpretation of such a result may be complicated by the presence of a cleavable signal peptide and glycosylation sites, which increase and decrease the molecular weight of a processed protein. There was no consensus signal peptide or cleavage site present within the deduced protein sequence, and no consensus sites for core glycosylation, leading to the conclusion that *in vitro* translated p64H1 was not glycosylated.

5.3 Determination that p64H1 is an integral membrane protein

Experimental data from Western blotting and immunofluorescence had suggested that p64H1 was a membrane associated protein. Translation *in vitro* in the presence of canine pancreatic microsomes, followed by centrifugation to pellet the microsomal vesicles, confirmed that p64H1 was associated with the microsomal membranes (Figure 5.1). It was not clear, however, if p64H1 was completely translocated across the microsomal membrane, partially translocated, or more loosely associated with the membrane. Translation as before, followed by vesicle extraction using a high pH buffer answered this question. After alkaline extraction, the radiolabelled p64H1 translation product remained associated with the microsomal membranes (Figure 5.1). This suggested that p64H1 was an integral membrane protein, partially translocated across the microsomal membrane. Such data required the presence of a hydrophobic transmembrane domain or domains within the amino acid sequence of p64H1.

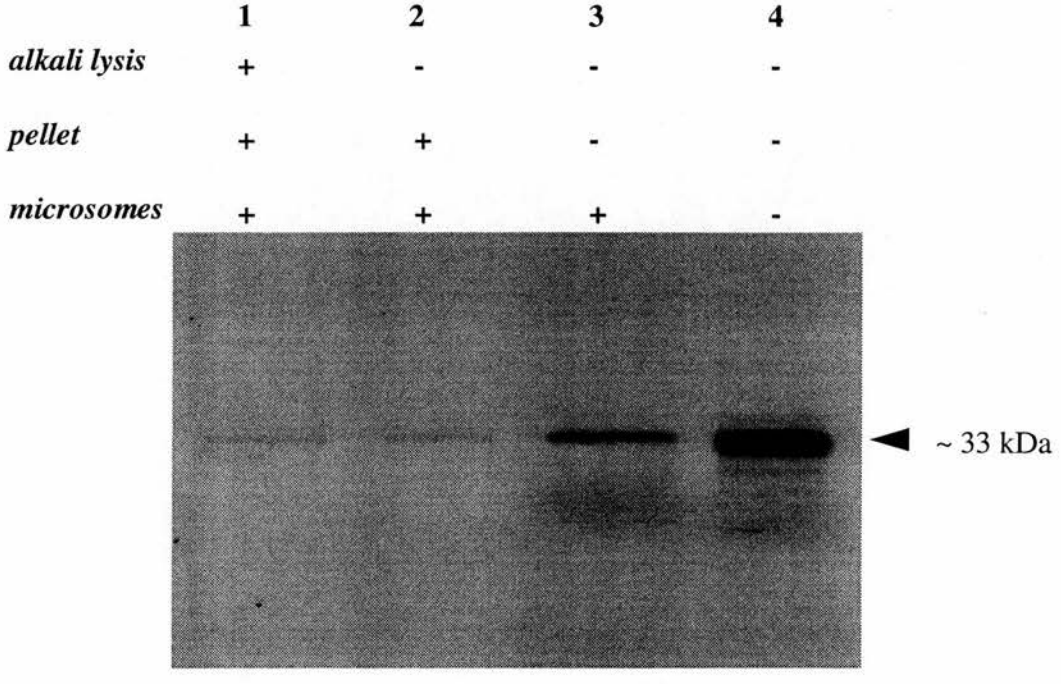


Figure 5.1 ***In vitro*** **translation of p64H1.** Autoradiogram shows *in vitro* translated p64H1. Lane 1: alkali disrupted microsomal membranes remained associated with the translation product; Lane 2: pelleted intact microsomes were associated with the translation product; Lane 3: Translation in the presence of canine pancreatic microsomal membranes did not produce a gel-shift, indicating an absence of glycosylation; Lane 4: translation in the absence of microsomes. The translation products were electrophoresed in a 10% SDS-polyacrylamide gel, which was dried and autoradiographed. The exposure time was 90 mins.

5.4 p64H1 possesses a single transmembrane domain

In order to probe the membrane topology of p64H1 translated *in vitro*, proteinase K was used to digest any protein domains exposed outside the microsomal vesicle. This proteinase protection assay removed the 33 kDa protein, but caused the appearance of a previously undetected 6 kDa species (Figure 5.2). Similar, parallel protection assays performed in the presence of 1% (v/v) Triton X-100 to disrupt the microsomal membrane completely removed radiolabelled products, confirming that the appearance of the 6 kDa species was due to protection of this domain from proteolysis. A Kyte-Doolittle hydropathy analysis for p64H1 (Figure 5.4) predicted a mainly hydrophilic protein, with a single potential transmembrane domain (Tm). Further analysis of the putative Tm domain, using the *PhD* algorithm (Rost *et al.*, 1995) predicted it to be in an α-helical conformation. The predicted molecular weight of the domain from the *N*-terminus to the *C*-terminal of the Tm domain was 6.2 kDa, in agreement with the hypothesis that p64H1 contained a single Tm domain, located towards the *N*-terminal of the protein (Figure 5.3).

5.5 p64H1 was phosphorylated *in vitro* by PKC

The native protein species which reacted with Ab990 had a molecular weight of 43 kDa. The deduced amino acid sequence of p64H1 had a predicted molecular weight of approximately 29 kDa, in agreement with the *in vitro* translated p64H1 product of apparent molecular weight 33 kDa. The difference in size between these proteins could not be accounted for by glycosylation. The deduced membrane topology of p64H1 predicts a short (approximately 6 kDa) domain within the

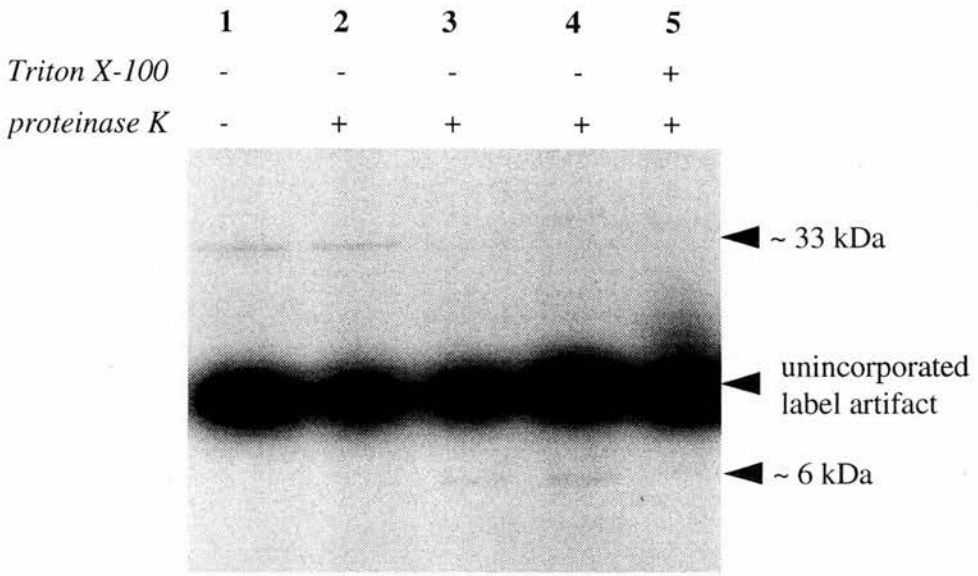


Figure 5.2 Determination of the membrane topology of p64H1 using *in vitro* translation and a protease-protection assay. Autoradiogram of *in vitro* translated p64H1, treated with Proteinase K and/or Triton X-100. Lane 1: p64H1 translated in the presence of canine pancreatic microsomal vesicles; Lane 2: as lane 1, but incubated for 10 mins with proteinase K (10 mg/ml); Lane 3: incubated with proteinase K for 20 mins; Lane 4: incubated with proteinase K for 30 mins; Lane 5: Incubated with proteinase K and Triton X-100 (0.1 %(v/v)) for 10 mins. The samples were electrophoresed in a 10% Tris-tricine SDS gel and exposed to autoradiographic film for 6 hours.

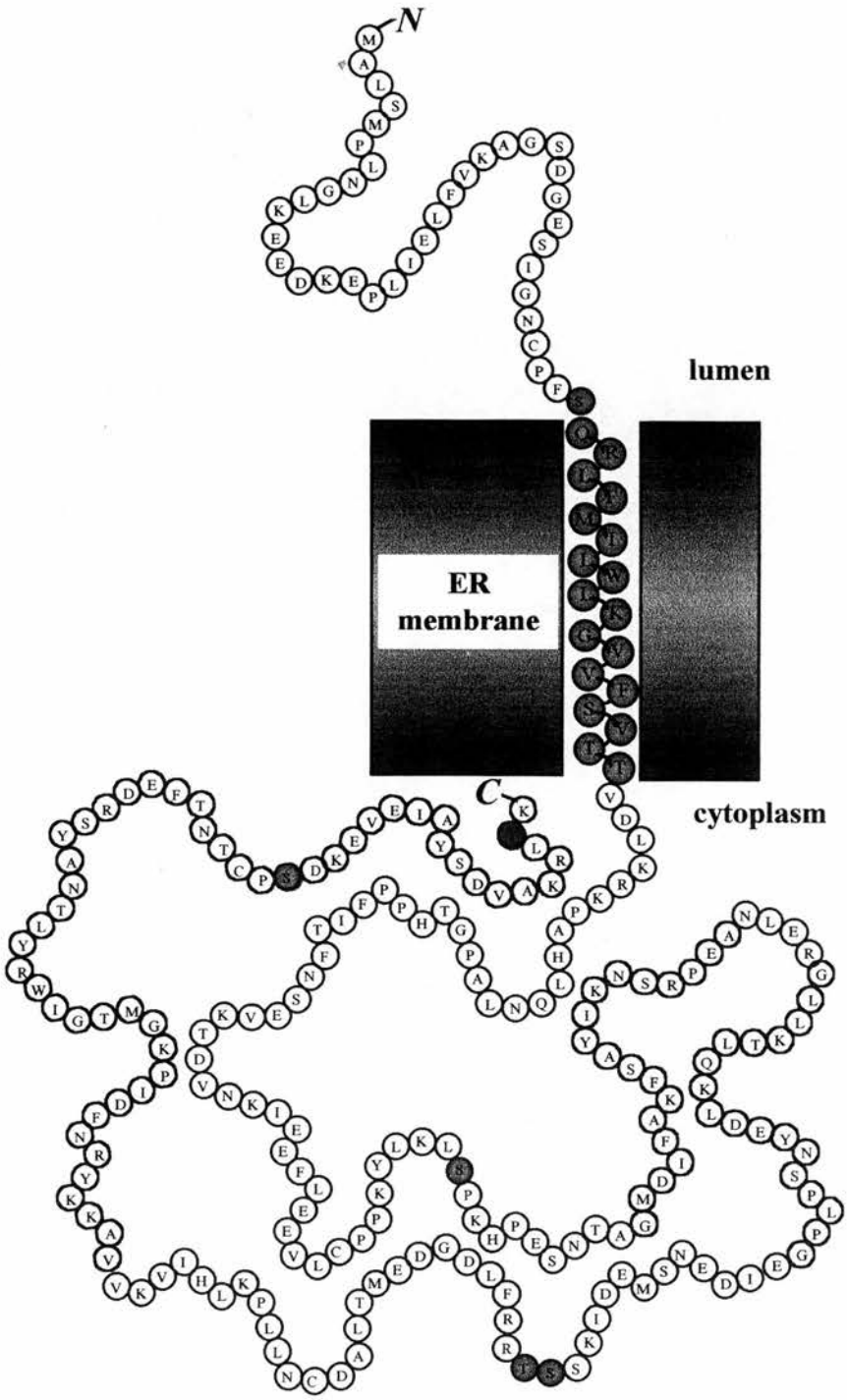


Figure 5.3 A diagrammatic representation of the predicted membrane topology of p64H1. The Tm domain is predicted to adopt an α–helical conformation. The PKC consensus phosphorylation sites are shaded in blue, and the PKA site in red.

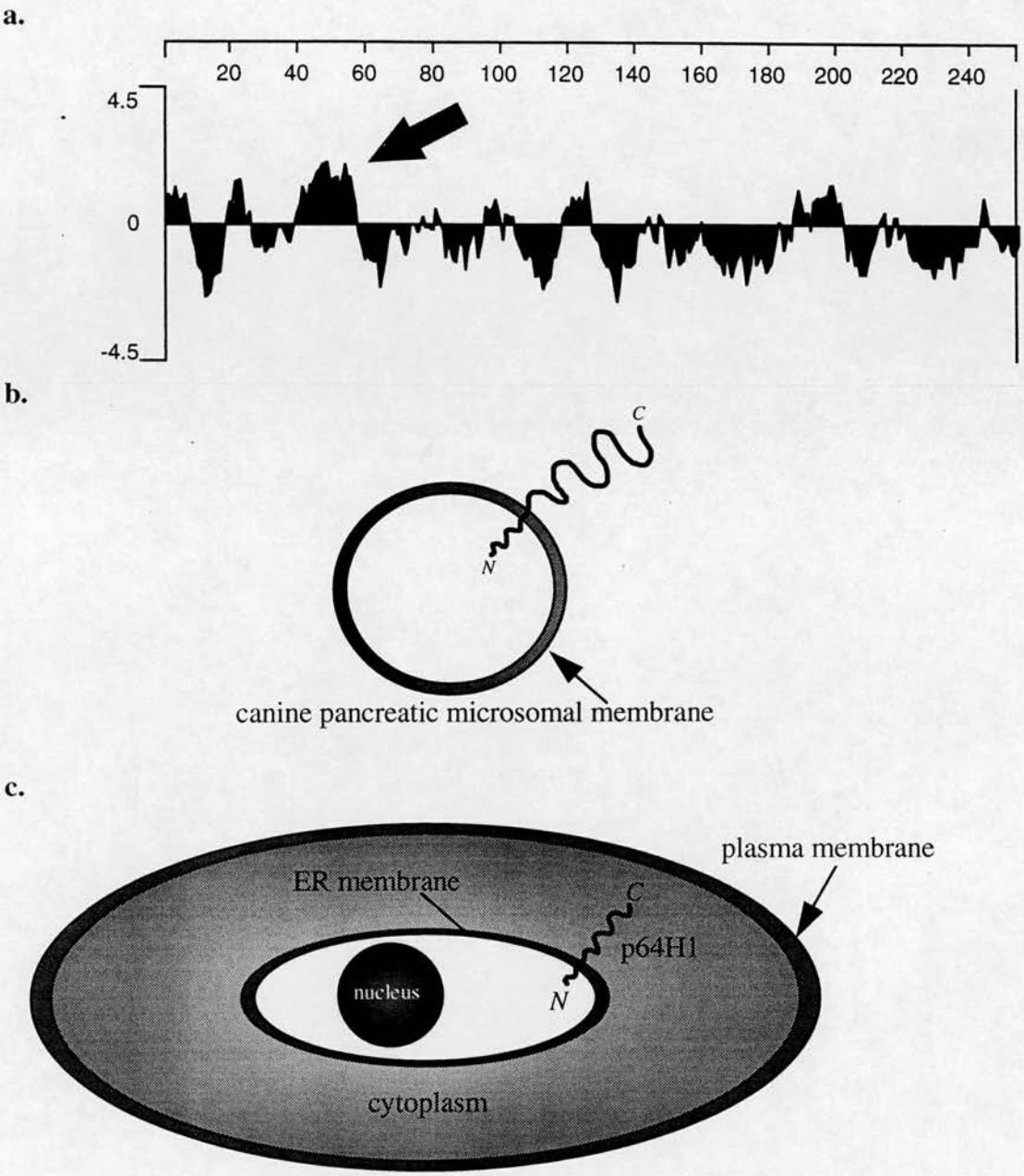


Figure 5.4 The membrane topology of p64H1 *in vitro* and *in vivo*. **a.** A Kyte-Doolittle hydropathy plot for p64H1. A window size of 9 amino acids was used. The predicted Tm domain is indicated by the arrow. **b.** The deduced topology of p64H1 within the canine pancreatic microsomal vesicle. **c.** The predicted membrane topology of p64H1 within the cell. The location of p64H1 within the ER membrane was demonstrated by indirect immunofluorescence and Western blot analysis, but the cytoplasmic location of the phoshorylated *C*-terminal domain would be preserved regardless of an intracellular or plasma membrane location for p64H1.

microsomal vesicle membrane bilayer, and a larger (approximately 25 kDa) domain residing outside the vesicle. This prediction, translated to an *in vivo* situation, meant that the larger domain would reside in the cytoplasm if p64H1 had an intracellular (ER or vesicular) or a plasma membrane location. A schematic illustration of the predicted membrane topology of p64H1 is presented in Figure 5.3. Several consensus phosphorylation sites were predicted to reside within the cytoplasmic domain, including 4 PKC sites, and 1 PKA site. As discussed previously, native p64H1 was enriched in regions of rat brain known to be rich in PKC (i. e. the hippocampus and the cerebellum). To examine PKC-mediated phosphorylation *in vitro*, microsomal vesicles, containing *in vitro* translated p64H1, were collected by centrifugation and resuspended in phosphorylation buffer. Aliquots were treated with rat brain PKC and analysed by Western blotting using Ab990. The apparent molecular weight of the protein recognised by Ab990 in immunoblot experiments increased from ~ 33 kDa in untreated samples to ~ 43 kDa in PKC-phosphorylated samples (Figure 5.5). In the treated samples, immunoblotting recognised a major species of ~ 43 kDa and a ladder of proteins from ~ 35 kDa to ~ 43 kDa. The additional proteins recognised may have represented different phosphorylation states of phospho-p64H1: the four cytoplasmically-disposed PKC-consensus sites could account for multiple phosphorylation states.

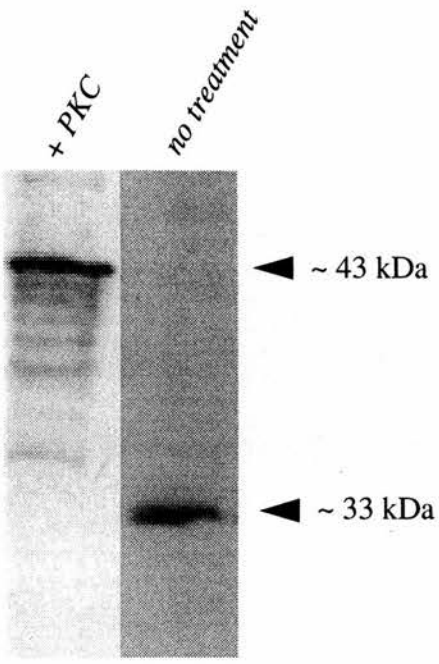


Figure 5.5 Western blot analysis of *in vitro* translated p64H1, before and after PKC-mediated phosphorylation *in vitro*. Aliquots were electrophoresed in a 10% SDS-polyacrylamide gel and blotted to a nitrocellulose membrane. p64H1 was detected using Ab990 and an HRP-conjugated goat anti-rabbit IgG second antibody. The reactive products were detected using ECL with an exposure time of 5 s.

5.6 Summary

A combination of predictive modelling and biochemical analyses demonstrated p64H1 to be a small (~ 30 kDa), non-glycosylated integral membrane protein. The deduced membrane topology of p64H1 suggested a single Tm domain, a short *N*-terminal domain, and a larger *C*-terminal domain. The *C*-terminal region of the protein was predicted to be cytoplasmic, regardless of cellular location, and contained multiple consensus phosphorylation sites for PKC and PKA-phosphorylation. Protein kinase-C-mediated phosphorylation was demonstrated *in vitro,* and caused the apparent molecular weight of p64H1 to increase from ~ 33 kDa to ~ 43 kDa.

Chapter 6

Towards the function of p64H1

6.1 Introduction

As discussed in the introduction, many ion channel proteins have been demonstrated to interact with other proteins, either as components of channel complexes (e. g. Barhanin *et al.*, 1996; Borsotto *et al.*, 1985; Changeaux, 1992; De Jongh *et al.*, 1989), or as part of a regulatory cascade (e. g. Jayaraman *et al.*, 1992; Wilson *et al.*, 1994; Egan *et al.*, 1992; Schwiebert *et al.*, 1995). The function of p64H1 remains unknown, despite a wealth of data regarding the expression, cellular and sub-cellular distribution of the mRNA and protein and its membrane topology. Attempts to elucidate the function of p64H1 are described in the following sections.

6.2 Single channel recording

Large-scale transfections were performed in order to determine whether p64H1 expressed in HEK293 cells affected endogenous Cl^- channels, or if p64H1 itself could give rise to Cl^- channel activity. Up to 20 100 mm plates of HEK293 cells were transfected with the plasmid pCI.p64H1 (Appendix I), and cultured for 48 h post-transfection. The cells were harvested, pooled, and P3 microsomal membranes were prepared from the total cell sample. Expression of p64H1 in the P3 fraction from transfected samples was confirmed by Western blotting (Figure 6.1). Parallel preparations made from cells transfected with the vector pCI alone contained no p64H1 detectable using Western blotting against these membranes. The P3 microsomal membranes were incorporated into voltage-clamped planar lipid bilayers in the presence of Choline Cl^- to select for Cl^- channels (Ashley, 1989; Hayman *et al.*, 1993). Anion channels were recorded from each of 7 independent transfections,

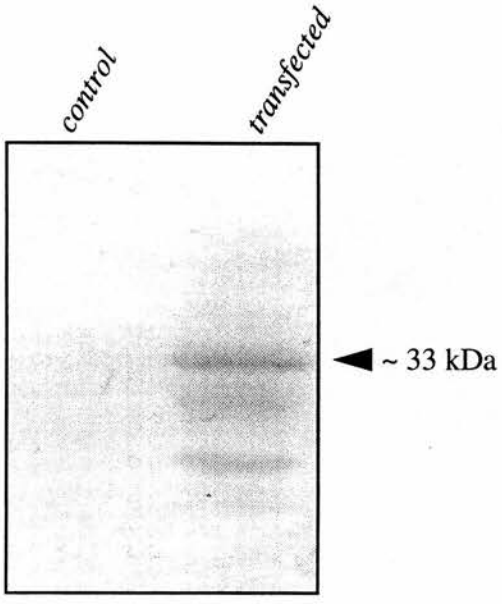


Figure 6.1 Detection of p64H1 in transfected HEK293 P3 membranes.

Western blot analysis using Ab990 demonstrating p64H1 in the P3 microsomal membrane fraction of transfected HEK293 cells 48 h after transfection. No p64H1 could be detected in cells which were transfected with the vector (pCI) alone in parallel control experiments. The reactive proteins were detected with an alkaline-phosphatase second antibody. Immune complexes were visualised using NBT/BCIP.

but not from 5 mock-transfected samples. The channels were unaffected by the addition of the indanlyoxyacetic acid derivative IAA-94 (up to 50 μM *cis* and *trans*), and they were not blocked by 5-nitro-2-(3-phenylpropylamino)-benzoate(NPPB) or 4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid (DIDS; up to 50 μM). All the electrophysiological experiments and analyses were performed by Dr. R. H. Ashley. These channels bore no resemblance to the ER channels previously reported (e. g. Clark *et al.,* 1997; Schmid *et al.,* 1988; Morier and Sauve, 1994).

6.3 p64H1 protein:protein interactions

p64H1 might represent endogenous channels normally present in intracellular membranes, or the presence of p64H1 may activate novel endogenous channels in brain ER. Further experiments were performed in an attempt to isolate interacting proteins associated with p64H1 *in vivo.*

Western blot analysis of native rat brain microsomal p64H1 electrophoresed in a Blue Native polyacrylamide gel system by Dr. S Howell demonstrated native p64H1 to have a molecular mass of approximately 130 kDa (Howell *et al.,* 1996). There are several possibilities that could have given rise to a native p64H1 protein complex of 130 kDa. p64H1 may form homotrimeric or homotetrameric multimers *in vivo,* or the protein may interact with other protein(s) to give rise to a heteromultimeric protein complex. Experiments performed by Dr. S. K. Mciver demonstrated that purified recombinant p64H1 binds to β-actin *in vitro.* There are several examples of recognised ion channel proteins which interact with actin *in vivo* and *in vitro,* (e. g. Levina *et al.,* 1994; Hug *et al.,* 1995; Cantiello, 1995; Cantiello and Prat, 1996;

Smith and Benos, 1996), and it remains to be seen if the p64H1-actin interaction is significant *in vivo*. The isolation and identification of proteins which interact with p64H1 *in vivo* would provide information which may reveal the function of p64H1. Attempts to isolate candidate proteins which may interact with p64H1 *in vivo* are described in the following sections.

6.4 The yeast two-hybrid interaction trap

6.4.1 Introduction

The yeast two-hybrid interaction trap (YTH) describes an *in vivo* assay which employs *Saccharomyces cerevisiae* reporter strains to detect protein:protein interactions (Fields and Song, 1989). The technique relies upon the fact that some eukaryotic transcriptional activators comprise discreet domains encoding a DNA-binding- (DB) and an activating- (AD) domain (e. g. GAL 4; Ma and Ptashne, 1987), which may be physically separated but nevertheless remain functional. The transcriptional activation activity of these proteins depends upon the recognition of specific upstream promotor sequences by the DNA-binding domain (the upstream activating sequence, UAS) and the recruitment of other transcription factors by the activating domain (Ma and Ptashne, 1987). Transcription is only initiated when both the DB and the AD are in close physical proximity. The YTH assay requires the creation of 2 fusion proteins: one is generated from GAL 4 DB and a "bait" protein of known identity, and the second from GAL 4 AD and an unknown protein. In this

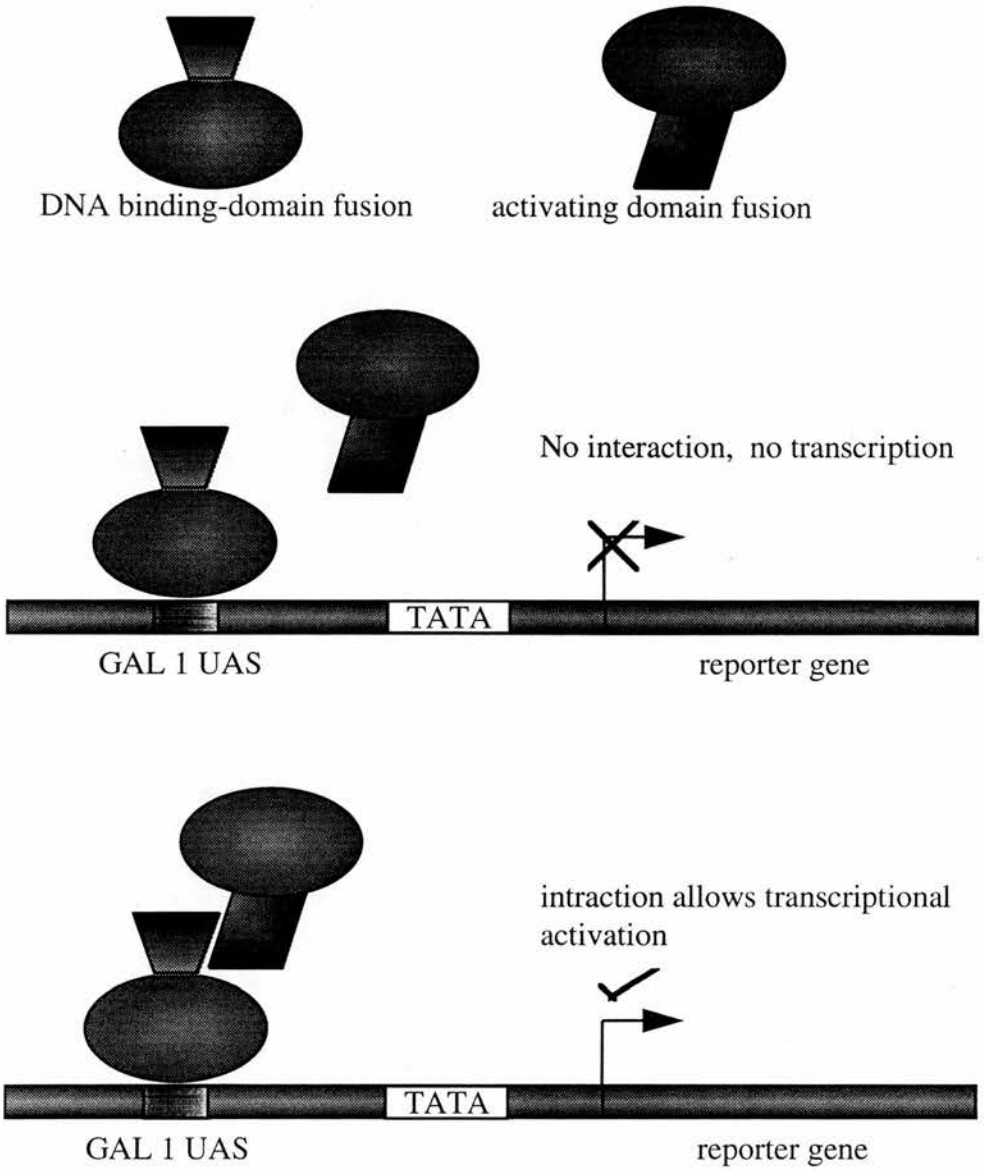


Figure 6.2 The yeast two-hybrid interaction trap. The specific interaction between a DNA binding domain fusion protein and an activating domain fusion protein allows the transcriptional activation of a reporter gene to occur. The lack of interaction means the reporter gene is transcriptionally silent. The activation of the reporter gene may be selected for, and yeast clones containing plasmids encoding both fusion proteins may be isolated.

way, a library of candidate proteins fused to GAL 4 AD may be generated (Chien *et al.*, 1991; Fields and Song, 1989). Plasmids containing inserts encoding both fusion proteins are introduced into specially-constructed *S. cerevisiae* reporter strains which are deficient in the GAL 4 protein, and which contain reporter genes (nutritional markers) under the control of promotors containing the GAL 4 UAS. If the "bait" protein and a fusion protein from the library interact, GAL 4 DB and GAL 4 AD will be brought into close molecular proximity, and transcription will initiate. The interaction between the fusion proteins can be detected by assaying for activation of a reporter gene (Figure 6.1).

The potential advantages of using a YTH screen to detect an interacting protein are numerous. The cDNA encoding the interacting protein (or domain) is available immediately for manipulation and identification. The procedure is potentially rapid, and recent YTH systems offer a range of screening assays to help eliminate false positives. The "bait" protein may consist of an entire protein or discreet domains or sub-domains, permitting the examination of exact interacting sequences within a larger polypeptide. The YTH assay was used to screen a rat whole brain cDNA library encoding candidate protein:AD fusion proteins for proteins which may have interacted with a p64H1:DB fusion protein.

6.4.2 Screening a YTH library with p64H1

6.4.2.1 Generating p64H1:GAL4 DB fusion proteins

A commercially prepared YTH library (Rat whole brain YTH library, Clontech) was screened with 2 plasmids encoding GAL 4 DB:p64H1 fusion proteins. The first

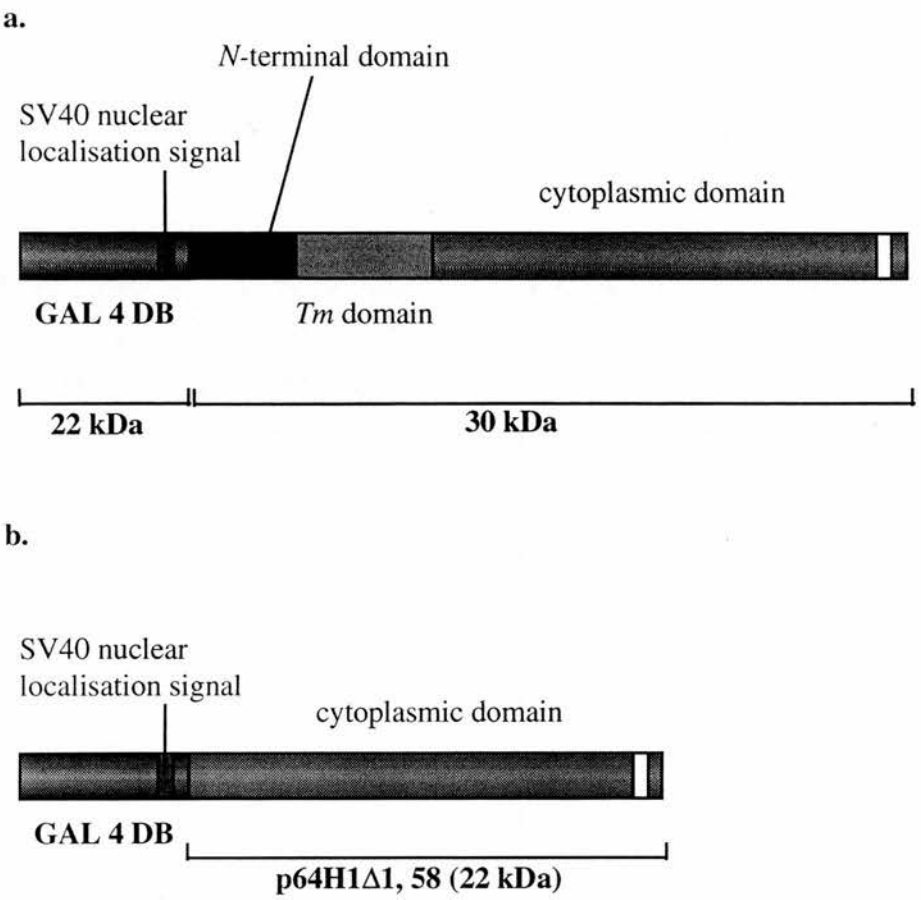


Figure 6.3 GAL 4 DB fusion proteins used to screen the YTH library.

a. Diagrammatic representation of the fusion protein encoded by pAS2-1.p64H1 (predicted molecular mass ~ 52 K). The white box near the *C*-terminal represents a putative ER-retention signal (KRXXX) within p64H1. **b.** Diagrammatic representation of the fusion protein encoded by pAS2-1.p64H1Δ1, 58. The protein is exactly the same as pAS2-1.p64H1 except the *N*-terminal 58 amino acids of p64H1 have been deleted resulting in the removal of the *N*-terminal and Tm-domains, leaving a fusion protein of predicted molecular mass ~ 44 K.

fusion protein vector was constructed by sub-cloning an *Nco* I-*Xba* I restriction fragment from pTAg.p64H1 into the vector pAS2-1, which encodes the *TRP1* auxotrophic marker gene. This restriction fragment contained the entire ORF of p64H1, and ~ 500 bp of 3'-UTR. The *Nco* I (CCATGG) site lay within the Kozak consensus initiating methionine motif (Kozak 1984 a, b and c), and the ligation generated an "in-frame" fusion with vector-derived sequences encoding the GAL 4 DB (pAS2-1.H1; Figure 6.2 and Appendix I). The successful detection of protein:protein interactions using the YTH assay relies upon the localisation of the interacting fusion proteins to the yeast nucleus (Fields and Song, 1989; Van Aelst *et al.*, 1993). To achieve this, the vector pAS2-1 contained an SV40 nuclear localisation signal to direct the "bait" fusions to the nucleus, although it has been suggested that some proteins which contain a Tm domain are not efficiently transported across the nuclear membrane (Van Aelst *et al.*, 1993). The second fusion protein construct was therefore generated to contain only the cytoplasmic domain of p64H1. A truncated form of *p64H1* cDNA was amplified using LDPCR using full-length *p64H1* cDNA as a template and oligonucleotide primers which annealed to the regions of cDNA encoding the *C*-terminal and an internal sequence after the proposed Tm domain ("forward": Y1318; 5-GATCGGATCCAACTGAAAAGGAAGCCTGCACAT-3'; "reverse": 5'-CACGGATCCCCTACTTGGTAAGTCTCTTGGCGAC-3'). Both primers contained a B*am*H I restriction endonuclease recognition site (GGATCC, underlined) and the "reverse" primer included the termination codon of p64H1 (double-underlined).

The LDPCR was performed as before, using the following thermal cycling conditions; 95°C, 1 minute, when 0.7 µl of *Taq/Pwo* polymerase cocktail were added, then 94°C, 30 s; 52°C, 30 s; 68°C, 45 s; for 30 cycles. The resulting PCR product (*p64H1Δ1, 58*) was ~ 650 bp long and encoded a truncated form of p64H1, lacking the *N*-terminal 58 amino acids of the full-length protein. This PCR product was cloned into the *Bam*H I site of pAS2-1, generating an "in-frame" fusion with vector-derived sequences encoding GAL 4 DB (pAS2-1.ΔH1; Figure 6.2 and Appendix I). The identity and integrity of the insert in pAS2-1 was confirmed by dideoxy sequencing as before.

6.4.2.2 Expression of p64H1:GAL 4 DB fusion proteins in *S. cerevisiae*

The pAS2-1.H1 and pAS2-1.ΔH1 vector constructs were transformed into the *S. cerevisiae* reporter strain CG1945 using standard procedures (Ito *et al.,* 1983; Schiestl And Gietz, 1989; Clontech YTH users manual), and transformants were selected on SD-Trp media. Single transformed colonies were cultured in SD-Trp liquid media and total protein extracts prepared according to the manufacturer's instructions (Clontech). Western blot analyses were performed using a mouse monoclonal anti-GAL 4 DB antibody (Clontech) according to the manufacturer's instructions. The expression of novel proteins of the predicted sizes could be detected for both p64H1:GAL 4 DB fusion proteins (Figure 6.3). Cells demonstrated to express p64H1 of p64ΔH1 did not grow on SD-Trp/-Leu/-His, indicating an absence of GAL 4 activation by the "bait" proteins. In addition, colonies cultured on SD-Trp were LacZ negative.

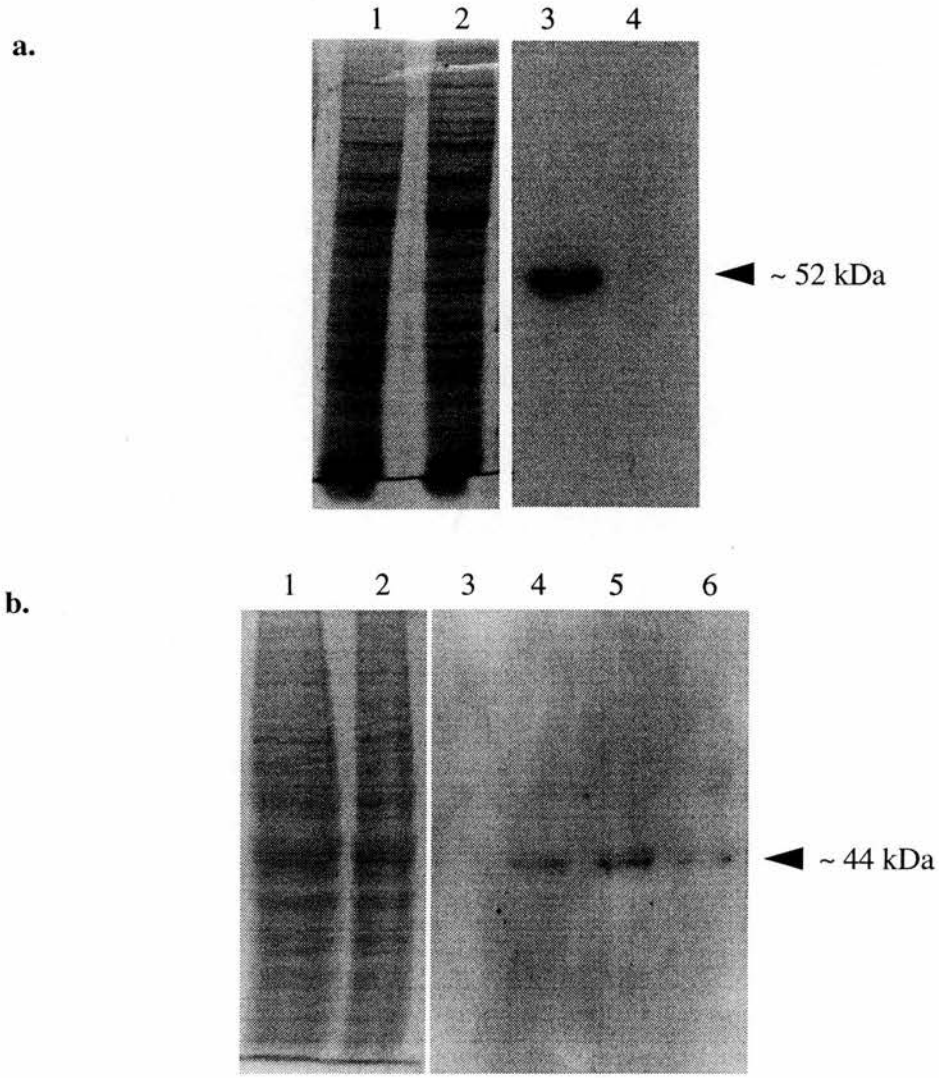


Figure 6.4 Expression of GAL 4 DB:p64H1 fusion proteins.
a. Detection of a GAL 4 DB:p64H1 fusion protein in protein extracts from transformed CG1945. Lane 1: transformed CG1945 extract, Coomassie stained; Lane 2: untransformed extract; Lane 3: Western blot detection of a protein of 52 kDa, the predicted size of the fusion protein; Lane 4: absence of fusion protein in untransformed extracts. **b.** Detection of the GAL 4 DB:p64H1Δ1, 58 fusion protein in protein extracts from transformed CG1945. Lane 1: transformed extract, Coomassie stained; Lane 2: untransformed control; Lane 3: no fusion protein detected in untransformed extract; Lanes 4-6: a fusion protein of ~ 44 kDa was detected in transformed extracts. Fusion proteins were detected using a monoclonal anti-GAL 4-DB antibody and a HRP-conjugated anti-mouse IgG second antibody. Immune complexes were revealed using ECL.

6.4.2.3 Screening the YTH library

The reporter strain used in this study (CG1945) contained dual reporter genes. The "bait" vector (pAS2-1) contained the *TRP1* nutritional marker, and the library plasmids (pGAD10) contained the nutritional marker *LEU2*. Interaction of fusion proteins would transcriptionally activate the first reporter gene, the nutritional marker *HIS3*. Interaction was detected by incubating the transformants on highly-selective SD-Trp/-Leu/-His media to select for cells co-transformed with both "bait" and library plasmids, and also expressing the product of the *HIS3* gene. In addition, the *HIS3* inhibitor 3-AT was added to the media to suppress "leaky" expression of this gene by CG1945. The second reporter gene (*LacZ*) was under the control of a different promotor to the *HIS3* gene, but remained transcriptionally silent in the absence of interacting proteins binding to GAL UAS. Any clones surviving the first-round of nutritional selection must also be $LacZ^+$ to satisfy all the criteria for true interactions.

The YTH library contained SD rat whole brain cDNA synthesised from oligo d(T) and random hexamer primers, cloned into the vector pGAD10, encoding proteins fused to GAL 4 AD (Appendix I). The *S. cerevisiae* strain CG1945 was transformed simultaneously with 1 mg of either pAS2-1.p64H1 or pAS2-1.p64ΔH1 and 0.5 mg of the library cloned in pGAD10. Transformants were selected for on SD -Trp/-Leu/-His/+25 mM 3-AT media at 30°C for 10 d. In addition, aliquots of the transformation were plated on SD-Trp, SD-Leu and SD-Trp/-Leu plates to check the transformation efficiencies of "bait" and library plasmids, and the co-transformation efficiency using both "bait" and library plasmid. After this incubation, any colonies

surviving the first-round of selection were picked to fresh SD -Trp/-Leu/-His/+ 25 mM 3-AT, and incubated for a further 4-5 d, before assay for activation of the *LacZ* reporter gene. Activation of the *LacZ* reporter gene was detected using an X-Gal colony-lift filter assay according to the manufacturers' instruction.

6.4.2.4 Results of YTH library screening

After two independent screening trials using the "bait" plasmid pAS2-1.p64H1, no clones survived the initial nutritional selection. A further library screen using the "bait" plasmid pAS2-1.p64ΔH1 yielded 27 clones which survived the initial round of screening. These colonies were picked separately to individual SD-Trp/-Leu/-His/ + 5 mM 3-AT plates and incubated for a further 7 days. None of these colonies were positive for LacZ expression.

6.5 Summary

The appearance of anion channels in the microsomal fraction of HEK293 cells transiently-expressing p64H1 which were not present in cells transfected with vector alone indicates that p64H1 is involved with anion transport within the cell. The demonstration that transiently-expressed p64H1 in HEK293 cells was localised to the membranes of the ER, and Western blot analysis detecting p64H1 in the P3 microsomal fraction of these cells supports the hypothesis that the anion transport recorded is due to the presence of p64H1 in these membranes. p64H1 was demonstrated to participate in protein:protein interactions *in vitro*, but screening a yeast two-hybrid library to identify candidate proteins was unsuccessful.

Chapter 7

Conclusions

7.1 Conclusions and discussion

The isolation of *p64H1*, a cDNA clone encoding an intracellular homologue of p64, was the first demonstration of an intracellular brain Cl^- channel protein, suggesting a family of p64-like proteins. The presence of *p64H1* mRNA and gene product was demonstrated in a variety of rat tissues using Northern hybridisation, immunoblotting, immunohistochemistry, mRNA *in situ* hybridisation and indirect immunofluorescence. Differences between p64 and p64H1 in primary sequence, localisation and membrane topology indicate that p64H1 is not simply the rat brain isoform of p64, but is a different protein. Northern blot analysis at reduced stringency revealed the presence of three transcripts which hybridised with the *p64H1* probe. It is possible that the largest of these species (~ 6.5 kb) encoded rat p64. Preliminary data provided by cDNA library screening at reduced stringency has allowed the isolation of a partial cDNA encoding another novel homologue of p64, *p64H2*, which is encoded by the smallest (~ 2.4 kb) transcript (R.R. Duncan and R.H. Ashley, unpublished data). Further experimental work will help to elucidate the function of this gene and its product *in vivo*.

The data obtained from these experiments showed *p64H1* mRNA to be approximately 4.3 kb in length. The clones isolated encoding p64H1 contained 753 bp of coding sequence, with 165 bp of 5'-UTR. The existence of additional 5'-UTR cannot be ruled out, but the discrepancy in size between the ORF and the full-length sequence may be accounted for by the presence of exceptionally long 3'-UTR sequences within this gene. Several cDNA clones isolated from the λZAP II rat olfactory bulb library contained such sequence. Although these flanking sequences

were not characterised fully, agarose gel electrophoresis and partial sequence analysis determined that they contained approximately 3.5 kb of 3'-UTR.

The developmentally regulated expression of *p64H1* may be significant as the rat cerebellum is one of the areas of the neonatal rat brain to develop significantly after birth (Jacobson, 1978). Purkinje cells originate on day 14 - 17 of rat embryonic development, then remain quiescent for several days. The Purkinje cells begin to differentiate rapidly after the granule cells have migrated past them in cerebellar development, 4 - 20 days after birth. A distinctive Purkinje cell layer is only apparent at approximately 4 days after birth (Altman and Winfree, 1977). The absence of detectable *p64H1* mRNA at day 16 of embryonic development and the low levels detected at day 20 (even though the amount of embryonic mRNA transferred to the membrane was greater than that in the adult sample) may reflect this late development.

Immunofluorescent staining showing that p64H1 protein was clearly localised to the nuclear envelope and ER of HEK293 and HT4 cells was supported by Western blot data which indicated that native rat brain p64H1 protein was contained within the P3 microsomal membrane fraction, with very little expression detected elsewhere. The P3 microsomal fraction has been shown to be enriched with ER membranes (e. g. Gray and Whittaker, 1962), suggesting that native p64H1 is localised to this compartment. These data combined support the hypothesis that p64H1 is an ER membrane-associated protein, and exclude the possibility that the immunofluorescence pattern seen is due to over-expressed, mis-folded protein being trapped in the ER. The ER-retention-signal found near the *C*-terminal of p64

(KRXXX; Redhead *et al.*, 1997) is also present in p64H1, and may function to localise p64H1 to the membranes of the ER. Levels of expression of p64H1 were reduced in the HT4 cell-line compared to the HEK293 cells as estimated from immunofluorescence. This may reflect lower expression directed from the CMV-promotor in HT4 cells in the absence of *trans*-activating factors present in HEK293 cells. The intracellular localisation of p64H1 within ER membranes is distinct from the secretory-vesicle localisation described for p64 (Redhead *et al.*, 1997). This may indicate a spatial separation of the proteins within the cell, suggesting a related function for a family of proteins.

Knowledge of neuronal ER is incomplete. Purkinje cells of the cerebellum are particularly enriched with markers for ER membranes, and in markers of intracellular Ca^{2+} stores (Villa *et al.*, 1992). Recent cytological information has shown the ER membranes of cerebellar Purkinje cells to adopt a peculiar stack-like formation of parallel smooth-ER cisternae, separated by evenly-spaced bridges (e. g. Villa *et al.*, 1992). Subsequently, it has been demonstrated that the formation of such ER cisternal stacks in transfected cells is dependent upon the presence of IP_3R protein within the ER membrane (Takai *et al.*, 1994). The precise cellular function of ER cisternal stacks remains unknown, but the formation of such structures has been shown to require full-length IP_3R (Takai *et al.*, 1994). Furthermore, it has been suggested that the cytoplasmic domains of the IP_3R form the dense projections which connect the cisternal stacks (Takai *et al.*, 1994).

Cerebellar Purkinje cells are also enriched in RyR protein. A recent analysis of RyR expression demonstrated that the "skeletal muscle" isoform is expressed

exclusively in the soma of cerebellar Purkinje cells, but the "cardiac" isoform has a widespread distribution within rat brain (Kuwajima *et al.*, 1992). In cardiac muscle, an influx of Ca^{2+} through the plasma membrane into the cytoplasm is required for the release of Ca^{2+} from the SR (Nabauer *et al.*, 1989). A novel, brain specific, voltage-gated Ca^{2+} channel has been demonstrated to be highly-expressed in cerebellar Purkinje cells (Mori *et al.*, 1991), and it has been suggested that this or another plasma membrane Ca^{2+} channel may couple with the "cardiac" RyR in the Purkinje cell (Kuwajima *et al.*, 1992).

Ryanodine receptors and IP_3 receptors co-exist in cerebellar Purkinje cell ER membranes (Walton *et al.*, 1991). These Ca^{2+} release channels appear to co-localise in the ER membranes in most regions of the Purkinje cell, but importantly they are differentially distributed in the dendritic processes, where only IP_3R has been found (Walton *et al.*, 1991). Evidently, the Purkinje cells of the cerebellum are well equipped and specialised for intracellular Ca^{2+} signalling, where the ER plays a central role. The differential localisation of the RyR and IP_3R within specialised ER membranes may indicate that Purkinje cells have multiple systems capable of regulating cytoplasmic Ca^{2+} levels in response to differential signals.

It would be of great interest to know whether or not the ER localisation of p64H1 within Purkinje cells reflects the localisation of either the RyR or IP_3 proteins. Immunolocalisation of p64H1 within Purkinje cells at the electron-microscopic level may reveal an interesting distribution of protein within the ER, and would allow the study of p64H1 within its native membranes. In addition, the function of p64H1 as a Cl^- channel protein (within a channel complex or otherwise) may be to provide

charge-compensation during the release of Ca^{2+} from intracellular Ca^{2+} stores. Investigating this hypothesis further would be difficult, but the generation and analysis of a p64H1 null-mouse would perhaps shed further light on the function of p64H1 *in vivo*. Further information regarding the cellular role of p64H1 *in vivo* may provided by the analysis of cerebella from mutant mice known to have defective cerebellar development and morphology.

Experiments based around *in vitro* expression directed by *p64H1* ORF cDNA showed p64H1 to be a non-glycosylated, integral membrane protein of ~ 33 kDa. p64H1 contained a single Tm domain, with a short, luminal *C*-terminal tail domain (~ 5 kDa), and a larger (~ 25 kDa) cytoplasmic domain. The apparent molecular weight of the native protein species (43 kDa) recognised by anti-p64H1 antiserum in rat tissue samples presented a question. The predicted molecular weight for the protein encoded by the *p64H1* ORF is ~ 29 kDa. The reasons for this size discrepancy may be multiple. The apparent molecular weight of the native protein recognised by immunoblotting may not be a true representation of the protein's actual molecular weight. There are several examples of proteins which migrate anomalously in SDS-PAGE; indeed, bovine p64 has an apparent molecular weight of approximately 64 kDa, but a predicted molecular weight of approximately 48 kDa (Landry *et al.*, 1993). *In vitro* phosphorylation by PKC caused the apparent molecular weight of the protein product to increase by ~ 10 kDa, to 43 kDa. These experimental data had several implications. It was previously predicted that bovine p64 had two, or possibly four, Tm domains, based solely on hydropathy plots (Landry *et al.*, 1993). The hydropathicity plots for p64H1 and the corresponding,

homologous *C*-terminal region of bovine p64 are very similar. The degenerate PCR primers used to amplify *BS2* from rat brain RNA were designed based upon the deduced amino acid sequence of bovine p64, corresponding to regions suggested to be membrane-spanning domains. One of these primers (M3044, FMILW) corresponded to sequence within the putative Tm domain of p64H1, but the "reverse" primer (M3045, DGDE) resided within the cytoplasmic domain.

In light of the experimental data demonstrating that p64H1 had a single Tm domain, the membrane topology prediction for bovine p64 seems unreliable. The predicted membrane topology for p64H1 would be conserved regardless of a plasma membrane, cytoplasmic vesicle membrane or ER membrane location *in vivo,* with the large, phosphorylated *C*-terminal domain residing in the cytoplasm. There was an additional consensus site for PKC-mediated phosphorylation located between the *N*-terminus and the Tm domain (Ser 38), which would reside intraluminally at the beginning of the Tm domain and may be non-functional *in vivo*. The molecular weight observed when *in vitro*-translated p64H1 was phosphorylated by PKC (43 kDa) corresponded to the apparent molecular weight of the native protein recognised by Ab990 in rat tissue homogenates. This suggested that native p64H1, recognised by the antiserum, was in a phosphorylated state. It may be significant that *p64H1* mRNA and protein products are localised in regions of the brain (i. e. the hippocampus and cerebellum) which were previously demonstrated to be enriched for PKC expression (Saito *et al.,* 1989; Ito *et al.,* 1990; Kose *et al.,* 1990; Kawasaki *et al.,* 1994). Non-phosphorylated (~ 33 kDa) protein was never detected in immunoblot analyses of rat tissues, suggesting that the non-phosphorylated state of

p64H1 in cells is rarely found, or that certain cellular conditions or factors are required to dephosphorylate p64H1. However, Western blot analysis of protein preparations from cultured cells transiently-transfected with an expression vector containing *p64H1* cDNA demonstrated the presence of a 33 kDa protein in transfected cells, but not in control samples. The presence of non-phosphorylated protein in this case may have reflected the heterologous expression of p64H1 in the absence of factors required for the normal regulation of p64H1.

Anion channels recorded from HEK293 microsomal membranes containing recombinant p64H1, and previously from p64-containing microsomal membranes, were not blocked by the addition of IAA-94 (Landry *et al.,* 1992). The underlying molecular basis of these currents is impossible to describe at present, but work already underway involving the expression and purification of glutathione-*S*-transferase-tagged p64H1 in *Sch. pombe*, carried out by Dr. J. Creanor, has yielded large amounts of pure recombinant p64H1. Further electrophysiological analysis using this purified protein may determine whether p64H1 itself can mediate Cl^- transport in a planar-lipid bilayer, or whether p64H1 requires the presence of other proteins to elicit Cl^- currents.

It cannot be determined at this stage whether p64H1 is an ion channel, a subunit of an ion channel (pore-forming or otherwise), or an activator of endogenous HEK293 microsomal anion channels. The difficulty in ascribing ion channel function to a heterologously-expressed protein is illustrated by the extensive literature describing minK as an independent ion channel (e. g. Wang and Goldstein, 1995; Tzounopoulis *et al.,* 1995), and the subsequent demonstration that minK is not an

ion channel itself, but an activator of another more conventional K^+ channel (L_vLQT1; Barhanin *et al.*, 1996, Sanguinetti *et al.*, 1996). It is safe, however, to conclude that the novel anion channels recorded in the above experiments are due in some way to the presence of p64H1 in the microsomal membranes of transfected HEK293 cells.

Attempts to isolate and identify cDNAs encoding potentially interacting proteins using a YTH assay proved unsuccessful. There are several possible reasons why the YTH assay was unfruitful. Firstly, p64H1 may not actually interact with any other proteins *in vivo*. This is unlikely in light of actin-binding studies and native gel analyses. Secondly, several criteria are required to be fulfilled in order for a YTH assay to be successful. The "bait" protein and the library protein must be efficiently expressed and directed to the yeast nucleus before any interaction can be detected. The expression of both p64H1 fusion proteins (directed by pAS2-1.p64H1 and pAS2-1.p64ΔH1) was demonstrated by Western blotting of transformed yeast proteins (Figure 6.5). However, the localisation of the fusion proteins to the yeast nucleus could not be confirmed. Previous reports have suggested that some membrane proteins are not directed to the yeast nucleus if the Tm domain or other targeting signals are present (Van Aelst *et al.*, 1993). Although pAS2-1.p64ΔH1 encoded a fusion protein lacking the Tm domain of p64H1, the putative ER *C*-terminal retention sequence described in bovine p64 (Redhead *et al.*, 1997) was present in the truncated p64H1 fusion protein. It cannot be determined at this stage whether this signal sequence could function to direct the fusion protein to the yeast ER in the absence of

a Tm domain, but further work using a YTH assay may benefit from the removal of both the Tm domain and the *C*-terminal region of p64H1.

Several other factors can influence the success of a YTH assay. Post-translational phosphorylation of p64H1 by PKC has been demonstrated *in vitro,* and it is likely that p64H1 is phosphorylated *in vivo.* Such modifications may be required for protein:protein interaction, but would not occur in *S. cerevisiae.* Finally, the hybrid proteins may have folded incorrectly, or the GAL 4 domains may have occluded the sites of protein:protein interaction. Such occurrences are impossible to detect, and may mean that some protein:protein interactions are impossible to detect using the YTH assay. A more "classical" biochemical approach (reviewed by Guarente, 1993) to detecting p64H1 protein:protein interactions may prove more useful.

The conclusion that bovine p64 is an ion channel (Landry *et al.,* 1989; Redhead *et al.,* 1992; Landry *et al.,* 1993; Redhead *et al.,* 1997) is unsafe. The evidence for this conclusion is summarised in the most recent paper describing work on p64 (Redhead *et al.,* 1997) as a co-purification of p64 with Cl^- channel activity, and the immunodepletion of Cl^- channel activity from bovine kidney microsomes using an anti-p64 antiserum. The possibility that p64 is an activator of another anion channel has not been addressed. The only single-channel data published ascribed to p64 activity (Landry *et al.,* 1989) showed recordings of three distinct anion channels with different conductance properties, recorded from the fusion of purified p64 with planar lipid bilayers. A reasonable explanation for the appearance of distinct anion channels would be the contamination of the purified p64 sample with other ion channel proteins, but this was never mooted. Immunoprecipitation studies using anti-

p64 antiserum to immuno-purify protein from solubilised kidney microsomes (Redhead *et al.*, 1992) detected a single 64 kDa species using Western blot analysis. A stained gel was not presented to demonstrate the absence of other proteins that may have co-immunoprecipitated with p64. In the absence of any other Cl^- channel protein with any structural or sequence similarity to p64, and of any definitive single-channel data, we cannot conclude that p64 alone is an ion channel.

More recently, a novel member of the p64-like channel protein family was described (NCC27; Valenzuela *et al.*, 1997). This protein is very similar to p64H1 in size and homology to the *C*-terminal half of bovine p64, but has a distinct intracellular localisation to the nuclear membrane. The proposed membrane topology of NCC27 suggests 2 Tm domains, but the deduced amino acid sequence is similar to that of p64H1, which has a single Tm domain. The description of NCC27 further supports the hypothesis that a family of intracellular p64-like proteins exists, and suggests that distinctive family members have different sub-cellular localisations.

The membrane topology deduced for *in vitro* translated p64H1 is unlike that of any other ion channel protein described to date, with the exceptions of phospholemman and Mat-8 (Palmer *et al.*, 1991; Moorman *et al.*, 1992; Morrison *et al.*, 1995). The structural similarities between these proteins and p64H1 are striking. Both have a very similar membrane topology to p64H1, and phosphorylation of phospholemman *in vitro* causes an increase in apparent molecular weight of approximately 10 kDa. Similar experiments using p64H1 increased the apparent molecular weight from 33 kDa to 43 kDa. Phospholemman and MAT-8 both cause the appearance of novel Cl^- currents when introduced into *Xenopus* oöcytes, which may be due directly to the

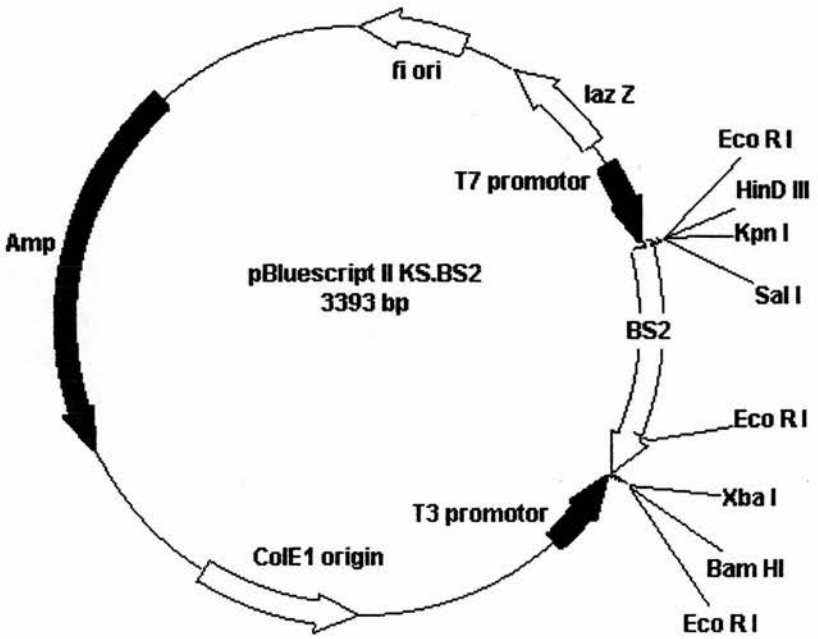
transport activities of these proteins, or indirectly due to endogenous channels being activated. It is interesting to speculate that phospholemman, MAT-8 and p64H1 may comprise a super-family of structurally related transport proteins, as previously suggested for MAT-8 and phospholemman (Morrison *et al.*, 1992). The extracellular and Tm domains of MAT-8 are homologous to those of phospholemman, but there is no sequence conservation between the cytoplasmic domains.

7.2 Concluding remarks and further research goals

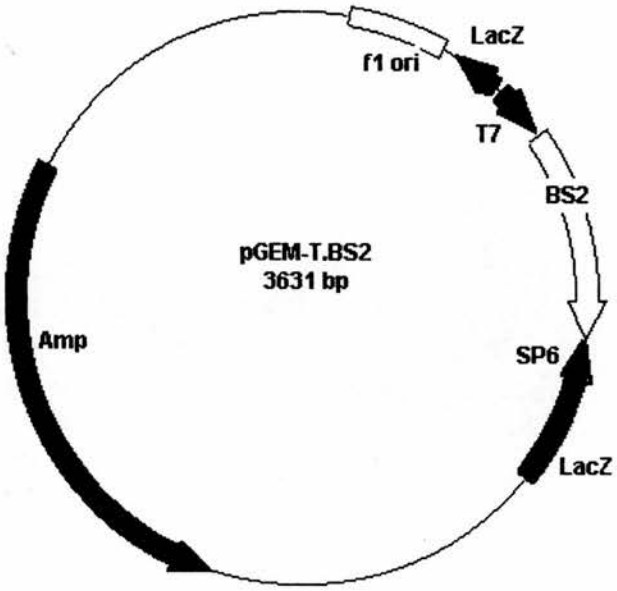
The function of p64H1 remains unknown, but it seems unlikely that p64H1 protein is a pore-forming ion channel subunit. Further work involving the electrophysiological analysis of pure, recombinant p64H1 may help to reveal the nature of the anion channels recorded from microsomal membranes containing p64H1. Preliminary work has already begun to express the cytoplasmic domain of p64H1 in *Sch. pombe,* with a view to purify enough protein to enable crystallographic studies and solve the 3-dimensional structure of this domain. This may provide information regarding the relationship of p64H1 with phospholemman and MAT-8, and may reveal the existence of a novel protein super-family.

Appendix 1

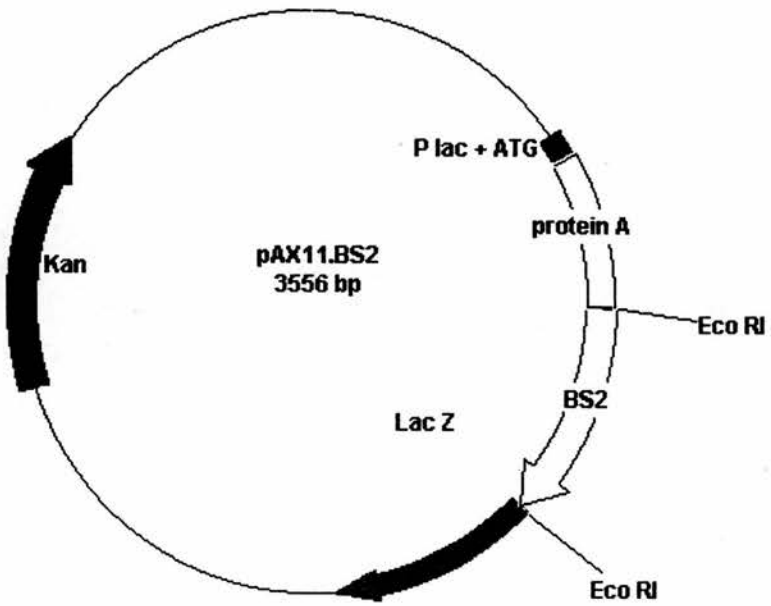
Plasmid Maps



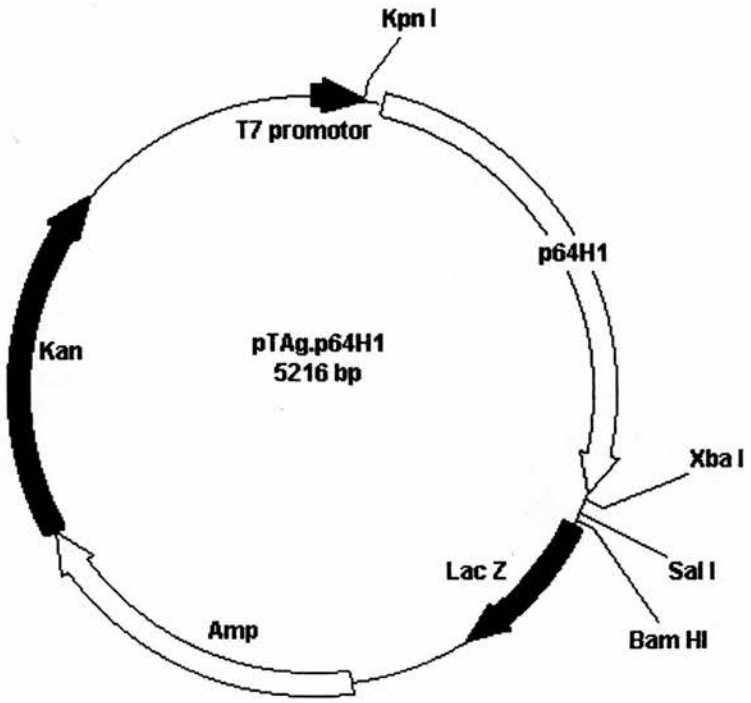
Plasmid pBluescript II KS.BS2. The *BS2* RTPCR-product was cloned into the *Eco*R I site of pBluescript II KS. The *Eco*R I restriction fragment was subsequently used as a probe in Northern hybridisations and to screen cDNA libraries.



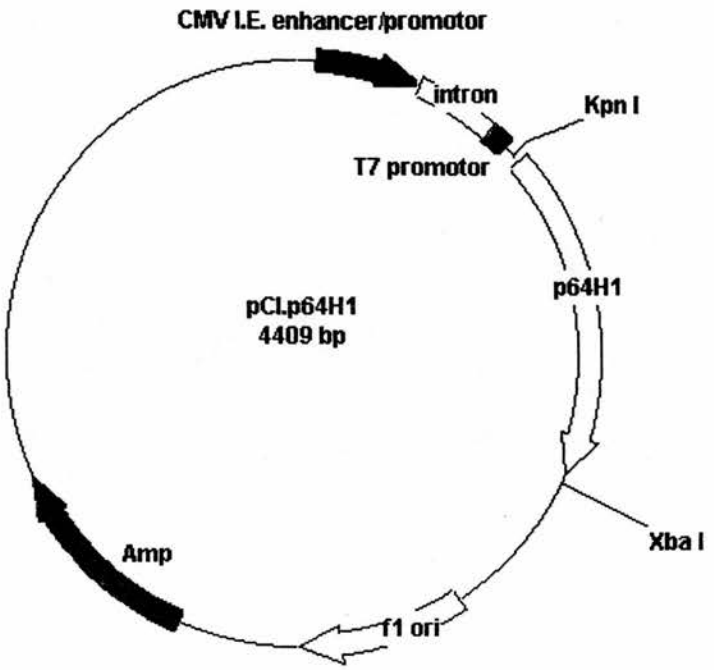
Plasmid pGEM-T.BS2. pGEM-T is a T/A-type cloning vector. The RTPCR-amplified *BS2* was cloned into the *Eco*R I site of pBluescript II KS, and found to contain an internal *Eco*R I site. The same RTPCR product was cloned into pGEM-T to confirm the identity of the PCR primers used, and the position of the *Eco* RI site.



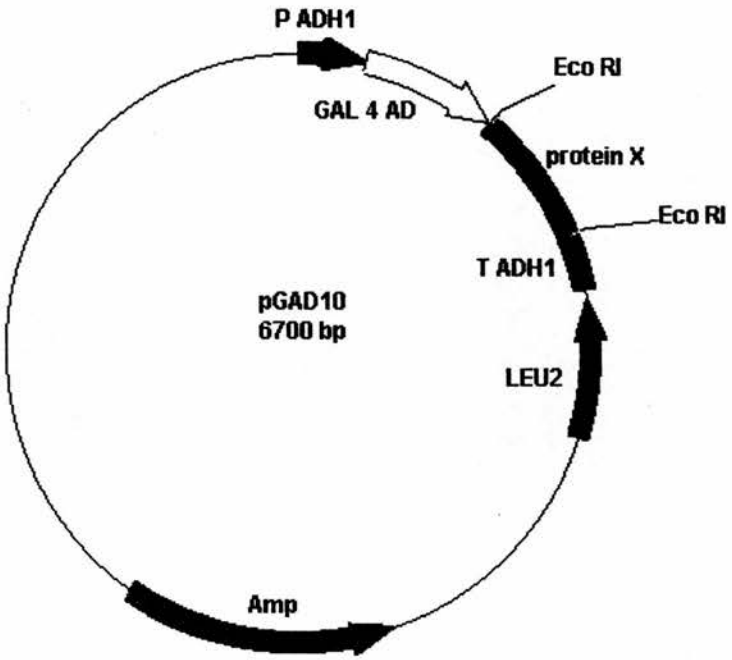
Plasmid pAX11.BS2. This plasmid contained the 342 bp *Eco*R I restriction fragment of *BS2,* cloned in-frame with sequences encoding *Staphylococcal* protein A, under the control of the IPTG-inducible *Lac* promotor. The fusion protein encoded by the cDNA insert was used as antigen to raise polyclonal antisera.



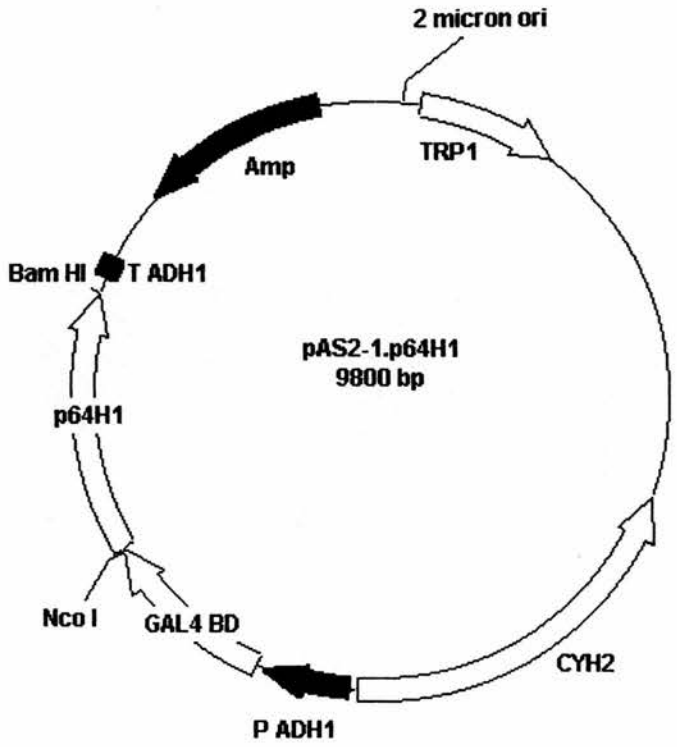
Plasmid pTAg.p64H1. This plasmid contained the cDNA insert isolated from λgt 10 clone λ 9, of ~ 1.4 kb. This contains 165 bp of 5'-UTR, and ~ 500 bp of 3'-UTR. This plasmid was used in *in vitro* translation experiments, and to sub-clone the *p64H1* insert into other vectors.



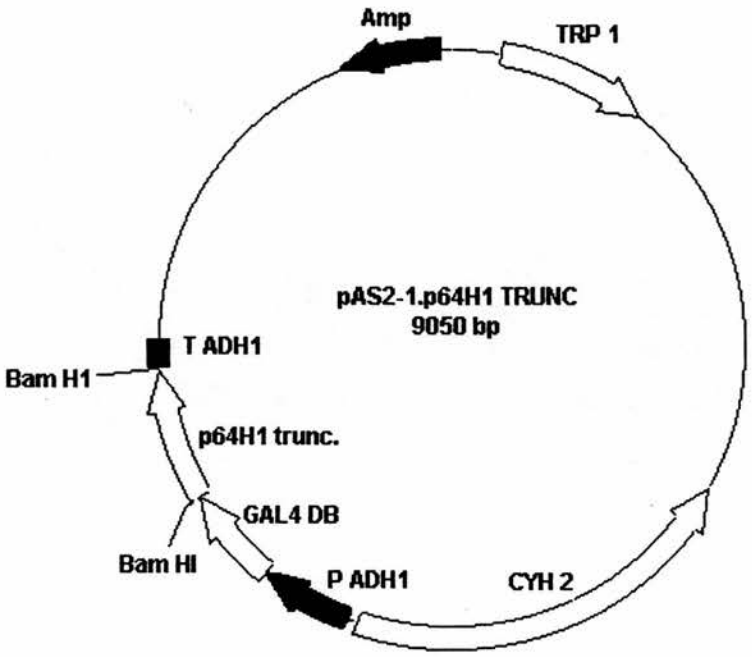
Plasmid pCI.p64H1. This plasmid contains a *Kpn* I-*Xba* I restriction fragment sub-cloned from pTAg.p64H1. This restriction fragment contains the entire coding region of *p64H1,* 165 bp of 5'-UTR, and ~ 500 bp of 3'-UTR. The plasmid also contains a chimaeric intron, reported to increase expression in some systems. The transcription of the insert is under the constitutive control of the CMV immediate-early enhancer/promotor element, and was used in transfection experiments involving mammalian cell-lines.



Plasmid pGAD10. This plasmid was used in the yeast two-hybrid library screen. The plasmid contains the nutritional marker *LEU2*. The library of rat brain cDNAs was cloned into the *Eco*R I site, fused "in-frame" to sequences encoding the GAL 4 AD.



Plasmid pAS2-1.p64H1 This plasmid contains an *Nco* I-*Bam*H I restriction fragment sub-cloned from pTAg.p64H1. The ligation generated an "in-frame" *N*- terminal fusion with the gene encoding GAL4 DNA binding domain. The insert contains no 5'-UTR, and ~ 400 bp of 3'-UTR.



Plasmid pAS2-1.p64ΔH1. This plasmid contains a PCR-generated truncation of p64H1, encoding a protein lacking the *N*-terminal and Tm domains (the *N*-terminal 58 amino acids). The insert was cloned into the *Bam*H I site of pAS2-1.

Appendix II

Publications

Abstracts

Ashley, R.H., Westwood, P.K. & Duncan, R.R. Cellular and intracellular localisation of a rat brain homologue of p64.(1997) Biophys. J. **72**, A160

Duncan, R.R. & Ashley, R.H. Molecular cloning and characterisation of a novel homologue of p64 from mammalian brain.(1997) Biophys. J. **72**, A160

Duncan, R.R., Silvestro, A.M. & Ashley, R.H. A p64 Cl^- channel-related gene is widely expressed in rat tissues, including brain.(1995) J. Gen. Physiol. **106**, 70

Howell, S., Duncan, R.R. & Ashley, R.H. Subunit composition of a rat brain p64 homologue.(1996) Progress In Biophysics & Molecular Biology **65**, PC315

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Howell, S., Duncan, R.R. & Ashley, R.H. Identification and characterization of a homologue of p64 in rat-tissues.(1996) FEBS Letters **390**, 207-210

Duncan, R.R., Westwood, P.K., Boyd, A. & Ashley, R.H. p64H1: expression of a new member of the p64 chloride channel protein family in endoplasmic reticulum. J. Biol. Chem. (1997). *in press.*

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