A transgenic mouse with PMP-22 directed GFP expression - a model for schwann cell behaviour

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In the peripheral nervous system the myelin sheath is produced by the spiral wrappings of the Schwann cell (SC) membrane around the axon. This provides insulation and increases the velocity of impulse propagation. The structure of myelin is maintained by a group of myelin proteins. Peripheral myelin protein-22 (PMP-22) is a 22 KDa glycoprotein, originally identified following nerve crush injury, that is found within SCs and is identical to the growth arrest specific protein GAS-3. The PMP-22 gene is regulated by two alternative promoters immediately upstream of two alternative non-coding exons. In order to study temporal and spatial expression of the PMP-22 gene and regulation of SC ensheathment and myelination, a transgenic mouse expressing the green fluorescent protein (GFP) driven by the myelin specific PMP-22 promoter was produced.

To achieve this the P1 promoter isolated from genomic DNA was initially incorporated into a plasmid containing the EGFP gene. *In vitro* transfection studies demonstrated appropriate expression of EGFP fluorescence. Microinjection of the transgene into pre-implantation fertilised embryos gave rise to three transgenic lines as confirmed by Southern blot and PCR. One founder expressed the transgene in a tissue specific manner. Mosaicism of expression both within an individual and between individuals was noted. *In vitro* manipulations showed that the expression patterns observed were independent of axonal contact and myelination but could be influenced by the extracellular matrix.

These GFP expressing transgenic mice potentially provide a means to determine the dynamics of SC-axon interactions during myelination and the behaviour of transplanted SCs into myelin deficient regions and the SCs response to injury.

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Declaration.

No part of this thesis has been submitted previously for a degree at this or any other institution. All the material is my own original work unless otherwise stated.

Abbreviations

B-Gal	Beta-galactosidase
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
bp	Base Pair
CAT	Chloroamphenicol Acetyl Transferase
C.I.P.	Calf Intestinal alkaline phosphatase
CMT	Charcot Marie Tooth Disease
CNPase	2',3'-cyclicnucleotide 3'-phosphdiesterase
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CR3	Complement type 3 receptor
Cx32	Connexin 32
DIC	Differential Interferance Copntrast optics
DMEM	Dulbecco's Modified eagles Medium
DRG	Dorsal Root Ganglia
DSS	Dejerine Sottas Disease
EDTA	Ethylenediaminetetraacrtic acid
EMP	Epithelial Membrane Protein
EtBr	Ethidium bromide
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FSH	Follicle Stimulating Hormone
Gal-C	Galactocerebroside
Gas-3	Growth Arrest Specific Protein 3
GDNF	Glial Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
HBS	HEPES buffered Saline
HBSS	Hanks Balanced Salt Solution

HCG	Human Chorionic Gonadotrophin
HIGS	Heat Inactivated Goat Serum
HMSN	Hereditary Motor and Sensory Neuropathies
HNMP-1	Hematopoetic and Neural Membrane Protein-1
HNPP	Hereditary Neuropathy with Liability to Pressure Palsies
IP	Interperitoneal
IPTG	β-D-isopropyl-thiogalactopyranoside
i.u.	International units
LB	Luria-Bertani broth
LH	Leutenising Hormone
MAG	Myelin Associated Glycoprotein
MBP	Myelin Basic Protein
N-CAM	Neural cell adhesion molecule
NCV	Nerve Conduction Velocity
NGF	Nerve Growth Factor
PBS	Phosphate Buffered Saline
p.c.	Post Coitus
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PEV	Position Effect Variegation
PLP	Proteolipid Protein
PMP-22	Peripheral Myelin Protein 22
PMS	Pregnant Mares Serum
PNS	Peripheral Nervous System
P_O	Protein Zero
SCIP	Suppressed cAMP Inducible POU Domain Protein
Shi	Shiverer mouse
Shi^{mld}	Myelin deficient mouse
TAE	0.04M Tris-acetate: 0.001M EDTA
T.E.	10mM Tris:1mM EDTA
Tr	*Trembler* mouse

TrJ	*Trember-J* mouse
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

Chapter 1.

Introduction

Chapter 1: Introduction

1.1 Glial Cells

The majority of nerve cells in the central and peripheral nervous system are surrounded by glia, the ratio of neurons to glia being in the order of 1 to 1 and they constitute half of the volume of the brain. Glial cells can be divided into different cell types by morphology, function and the cell surface antibody repertoire that they display. They perform a variety of functions including neuronal guidance during growth and development, maintaining the delicately balanced fluid environment of the neurons including the blood brain barrier, and a potential role in the immune response of the central nervous system has been suggested. One of the most important functions of any glial cell is the generation of the myelin sheath that insulates the axon allowing propagation of the action potential. Below is a brief description of a number of different glial types, their classification and potential roles in the nervous system.

1.1.1 Astrocytes - a versatile cell type

Astrocytes are one of the most common glial cell types found in the central nervous system (CNS) and their potential functions are varied. They can be readily identified with an antibody against the intermediate filament glial fibrillary acidic protein (GFAP) (Bignami *et al.,* 1972; Dahl and Bignami, 1985). Two distinct subpopulations of astrocytes have been identified *in vitro*, designated type I and type II astrocytes, which can be identified by their distinct morphologies, the cell surface markers they express and their growth kinetics (Raff *et al.*, 1983a; ffrench–Constant and Raff, 1986).

The variation between the two cell types reflects different developmental origins of each. Type I astrocytes that appear from birth are unable to differentiate into type II astrocytes that are present from the second post-natal week (Raff et al., 1983a). However the type II astrocytes were found to share a common precursor with oligodendrocytes, the oligodendrocyte precursor cell (Raff *et al.*, 1983b; Raff *et al.*, 1989).

A common role for cells of this lineage was suggested by the observation that type II astrocytes *in vitro* contribute to the node of Ranvier, extending processes that encircle the exposed axonal plasma membrane the region between adjacent myelin internodes formed by the oligodendrocytes (ffrench -Constant and Raff, 1986; Waxman, 1986).

Astrocytic processes are also found to ensheath brain blood capillaries and can induce tight junction formation in endothelial cells, maintaining the blood brain barrier (Tao-Cheng *et al.*, 1987). Secretion of growth factors and cytokines that are modulators of the immune system and the expression of class II antigens of the major histocompatibility complex also suggest a role in the defense of the nervous system for this glial cell type (Duchen *et al.*, 1992).

1.1.2 Radial Glia - neuronal guidance

During the development of the brain migrating neurons were first seen travelling along the fibres of glial cells. Glial guidance mechanisms have since been shown to provide a means of positioning maturing neurons at their correct location within developing structures (Rakic, 1971; 1972). Once neuronal migration is complete the majority of radial glial cell's differentiate into astrocytes (Levitt and Rakic, 1980). Transitional forms, as the cell's progress from bipolar radial cells to stellate astrocytes can be identified with monoclonal antibodies. The exceptions are the Bergmann glial cells in the cerebellum and Muller cells of the retina that maintain their radial structure in the adult brain (Suarez *et al.*, 1995).

The process of neuronal migration along glial fibres has been extensively studied in the cerebellar system where granule cells migrate along Bergmann glial fibres (Hatten *et al.*, 1984; Edmondson and Hatten, 1987). The soma of the bipolar neuron becomes closely apposed to the glial cell arm and a specialised junction is observed between them (Gregory *et al.*, 1988), a leading process is extended in the direction of movement. Neuronal filopodia and lamellipodia are seen to extend in many directions, but the driving force for migration is the neuron-glia apposition at the neuronal soma (Hatten *et al.*, 1984; Edmondson and Hatten, 1987; Gasser and Hatten, 1990).

The pattern of migration is remarkably similar in other regions of the brain and heterotypic cultures of cerebellar and hippocampal neurons and glia have demonstrated the commonality of the mechanism (Gasser and Hatten, 1990). It has been proposed that the interaction between the neurons and glia is mediated by the neural ligand astrotactin (Hatten 1990).

1.1.3 Microglia – immune responsive glial cells

Microglia were first described by del Rio Hortega (1932) in silver stained preparations for the light microscope. They have a characteristic morphology, with long branched and crenellated process and are found throughout the CNS. Monocytes generated from bone marrow circulate in the blood and are delivered to their target tissue where they differentiate further. During embryonic development monocytes can be seen to enter the retina and brain, where they differentiate into microglia (Austyn and Gordon, 1981; Hume *et al.*, 1983; Perry *et al.*, 1985).

Naturally occuring cell death during development is thought to attract circulating microglia to the site, indicating a possible role in tissue modeling. Additionally they have been shown to secrete factors that can stimulate angiogenesis and gliogenesis, processes that occur both pre- and post-natally (Perry and Gordon, 1988).

Cell surface antigens found on adult microglial cells include that of the complement type 3 (CR3) receptor (Perry *et al.*, 1985). Other components of the immune system have been identified. For example class II antigens of the major histocompatibilty complex are important in the activation of T helper lymphocytes. In certain pathological conditions microglia have been shown to express class II antigens on their surface, as have astrocytes (Fontana *et al.*, 1984; Lampson, 1987). The two cell types also share the ability to secrete a number of products common to macrophages (Fontana *et al.*, 1982). In response to injury of the brain circulating monocytes are recruited to the site of trauma, however the proportion that are resident microglia is difficult to determine (Lindsey *et al.*, 1986).

1.1.4 Myelin forming cells

One of the most important roles of glial cells in both regions of the nervous system is the production and maintenance of the myelin sheath. In the central nervous system this is achieved by the oligodendrocyte that can extend many processes, forming multiple internodes on different axonal processes. The Schwann cell of the peripheral nervous system forms a one-to-one relationship with an axon, forming only a single internode. More detailed discussion of these two cell types is found elsewhere (see section 1.2 and

1.3 and B1.1). Myelin is formed by the spiral wrapping of the oligodendrocyte or Schwann cell plasma membrane around the axon. The cytoplasm is then extruded, and the structure of compact myelin is maintained by a specific set of myelin proteins (section 1.1.5).

The myelin sheath is not continuous along the length of the axon, between each myelin internode is found a node of Ranvier. The overlapping membranes that build up the myelin sheath at the internode constitute a region of high resistance, restricting current flow through this area. Despite the support of the node in the central nervous system by astrocytic processes, lower resistance at the node of Ranvier means the action potential can flow through the neuronal membrane at this point. In this way the current is passed in a saltatory manner along the nerve fibre (Tasaki, 1939; Huxley and Stampfli, 1949). The impulse jumps from one node to the next, greatly increasing the rate of nerve conductance velocity.

The structure of the node of Ranvier and its role in mediating the axonal signal is complemented by the expression pattern of the axonal Na^+ channels that generate the action potential. Studies have shown an intrinsic difference in the membrane properties of the axon beneath the myelin internode and at the node of Ranvier. A high density of Na^+ channels is seen at the node both by cytochemical investigations and immunolabelling. It has also been confirmed by radiolabelled toxin and electrophysiology studies (Waxman and Ritchie, 1985). This distribution ensures that the channels are in the correct position relative to the resistance of the myelin sheath and that they are concentrated in such numbers as to enable propagation of the signal. Other potential advantages arise from this organisation. Fewer ions will enter and leave the axon as they are restricted to the node, in turn this limits the metabolic expenditure required to return the membrane potential to its resting state.

1.1.4.1 Formation of the node of Ranvier

The clustering of Na^+ channels that is crucial for successful conduction of the action potential can be seen to occur before the formation of the node of Ranvier. The mechanisms and interactions between neurons and glia that regulate this phenomenon are still disputed.

A number of reports describe the clustering of Na^+ channels without direct contact between neurons and glia. Kaplan *et al.* (1997) for example show that the culture of optic nerve glia beneath retinal ganglion cells can induce clustering along the retinal neurons, and that this capacity is restricted to oligodendrocytes. In contrast in the sciatic nerve Schwann cell contact with axons and myelination have been shown to be required for the aggregation of channels (Joe and Angelides, 1992; Dugandzija-Novakovic *et al.*, 1995; Vabnick *et al.*, 1996;). Rasband *et al.* (1999) have shown that *in vivo* in the CNS oligodendrocytes immunoreactive for early myelin proteins are present before Na^+ channel clustering begins. In the *shiverer* mouse, in which the primary phenotype affects Schwann cell behaviour, fewer and abnormal Na^+ clustering is seen (Rasband *et al.*, 1999).

1.1.4.2 Structure of a Myelinated Axon

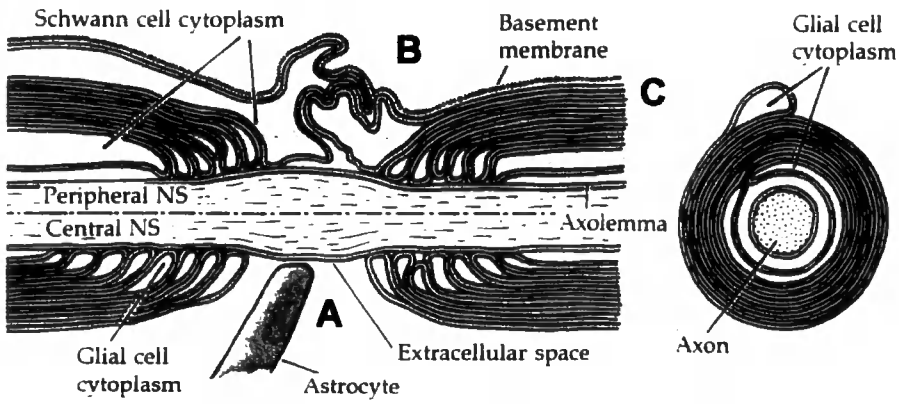
A schematic representation of myelin is shown in figure 1.1.4.2a, the central axon is surrounded by many wrappings of the myelin membrane. Electron micrographic images of peripheral myelin show the Schwann cell body and the inner leaflet in apposition to the axon (Figure 1.1.4.2b). At higher magnification each membrane layer can be clearly seen (1.1.4.2c). Below are descriptions of a number of structures associated with different regions of the myelin sheath.

Schmidt-Lanterman Incisures: Regions of uncompacted myelin found in the compact myelin of the peripheral nervous system. They contain significant amounts of cytoplasm (Ghabriel and Allt, 1981). The incisure spirals radially producing a continuous channel from the inner or adaxonal cytoplasm to the outer or abaxonal cytoplasm (Friede and Samorajski, 1969).

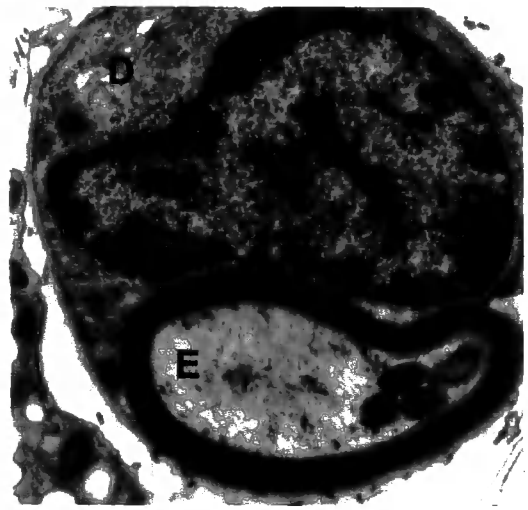
Paranodal Loops: Sections of uncompacted myelin adjacent to the nodes of Ranvier. These contain a helical rim of cytoplasm that connects the adaxonal and abaxonal domains. A number of desmosome like structures and tight junctions are found in the paranodal region and the lateral margins of the Schmidt-Lanterman incisures (Schnapp and Mugnaini, 1975; Shinowara *et al.*, 1980; Ghabriel and Allt, 1981). K^+ channels are

Figure 1.1.4.2 Representations of Myelin.

a. Schematic representation of the myelin sheath in both the peripheral and central nervous systems. A denotes the node of Ranvier, B is the paranodal region as the internode ends and C shows a region of compact myelin. **ai**. transverse section, **aii**. Cross sectional view. From Nichols *et al.* 1992 **b.** 34,000x electron micrograph of peripheral myelin. The Schwann cell body (D) is evident with multiple layers of membrane encircling the axon (E). **c.** 340,000x magnification electron micrograph of the myelin sheath. Individual lamellae of compact myelin can be seen.


Schwann cell cytoplasm
B
Basement membrane
C
Glial cell cytoplasm
Peripheral NS
Central NS
Axolemma
A
Glial cell cytoplasm
Astrocyte
Extracellular space
Axon

ai. **aii.**


D
E

b.

c.

found in this region of the Schwann cell plasma membrane, and are likely to be involved in K^+ buffering at the node.

Structural Artifacts: electron dense structures representing the apposition of the intracellular and extracellular membranes of the myelin membrane as it wraps around the axon are termed the **major dense line** and the **intraperiod line** respectively. These structures are maintained by specific sets of proteins.

1.1.5 Myelin Proteins

The majority of the dry weight of myelin is composed of many different classes of lipid (Inouye and Kirschner, 1988). Whilst some species are enriched in the myelin membrane over other plasma membranes none are specific to the myelin sheath. This is in contrast to the myelin proteins that are unique to myelin and integral to the formation of a functional sheath.

Connexin 32: Connexins are membrane spanning proteins that assemble to form gap junctions that facilitate the movement of ions and small molecules from cell to cell. Connexin 32 (Cx32) is found in both the central and peripheral nervous systems, in addition it can be localised to the liver and epithelial cells (Bennett *et al.*, 1991).

In the CNS, Cx32 is found in the cell bodies and processes of oligodendrocytes, but not in compact myelin. In the peripheral nervous system it displays similar temporal expression patterns to other PNS myelin proteins, and nerve crush results in its loss and recovery following axonal regeneration (Scherer *et al.*, 1995). Peripheral expression of Cx32 is restricted to the paranodal region and Schmidt-Lanterman incisures of compact myelin (Bergoffen *et al.*, 1993; Scherer *et al.*, 1995). These junctions are thought to form a radial pathway across the myelin membrane that is significantly shorter than the circumferential pathway through the Schwann cell cytoplasm (Scherer *et al.*, 1995).

In non-myelin forming Schwann cells Cx32 immunostaining is punctate and discontinuous, and is found in significantly less abundance than in the myelinating phenotype (Scherer *et al.*, 1995).

Myelin Associated Glycoprotein: Myelin associated glycoprotein (MAG) is found co-localised with Cx32 both in the PNS at the Schmidt-Lanterman incisures and the paranode, and the CNS in oligodendrocytes. In addition it is found along the adaxonal surface of myelinating Schwann cells in apposition to the axon, where it may mediate Schwann cell axon interactions. MAG is absent from regions of compact myelin. The presence of immunoglobulin like domains on the extracellular surface of the protein (Salzer *et al.*, 1987) and the L2/HNK epitope on the carbohydrate component (Kruse *et al.* 1984) suggest cell adhesion as the role for MAG. However it has not been found to form homophilic interactions and is thought unlikely to maintain the structure of the paranode and Schmidt-Lanterman incisures. This is more likely to be achieved by E-cadherin, a cell adhesion molecule that can interact homotypically and serve to hold the layers of uncompacted myelin together (Fannon *et al.*, 1995).

E-cadherin: E-cadherin is an adhesive glycoprotein that is restricted in its expression to the non-compacted regions of peripheral myelin. It is excluded from compact myelin and so has no role in the adhesion or compaction of the sheath. Uniquely to the cadherin family it mediates adhesion between membranes of the same cell. It is found at the Schmidt-lanterman incisures and paranodal loops where it forms junctions across the membranes, maintaining the structure of the non-compacted myelin (Fannon *et al.*, 1995).

Protein Zero: Protein zero (P_O) is a 28-30kDa protein exclusive to peripheral myelin where it constitutes approximately 50% of the total myelin protein (Greenfield *et al.*, 1973; Lemke and Axel, 1985; Lai *et al.*, 1987). P_O is an integral membrane protein with a single membrane spanning domain. The extracellular domain has a primary sequence characteristic of the immunoglobulin family (Lai *et al.*, 1987; Lemke *et al.*, 1988), it also carries the L2/HNK epitope common to cells involved in adhesion (Bollensen and Schachner, 1987).

P_O molecules form tetramers in the plane of the plasma membrane (Shapiro *et al.*, 1996). This functional unit interacts with tetramers of the opposing myelin membrane holding the compact myelin together (Filbin *et al.*, 1990; D'Urso *et al.*, 1990). The importance of

this level of structural support for the myelin sheath is seen in the shiverer mouse. This mouse mutant lacks the family of myelin basic proteins. They show severe loss of myelin in the central nervous system, yet the absence of MBP in the peripheral myelin does not affect myelin formation. It has been proposed that in the PNS compaction is maintained by another myelin protein that is absent from the CNS, namely P_0.

A recent report has also identified interactions between P_0 and another myelin protein, peripheral myelin protein 22 (PMP-22). Immunoprecipitation with antibodies to either protein brings down both myelin proteins. When cells transfected with one of the proteins were cultured with cells expressing the other, the proteins were recruited to and co-localised at apposing membrane surfaces (D'Urso *et al.*, 1999).

The intracellular domain of the P_0 molecule may also be of functional importance. It is highly charged and may stabilise interactions between phospholipid head groups of the myelin membrane at the major dense line (Lemke, 1988; Ding and Brunden, 1994).

The P_0 null mutant mouse (Giese *et al.*, 1992) is characterised by a severe but incomplete loss of peripheral myelin. However the correct association of Schwann cells and axons in the null mouse suggests that P_0 is not necessary for the initial interactions of myelin formation but that its main role is to maintain the structure of compact myelin.

Myelin Basic Proteins: This family of proteins range in size from 14 to 23kDa. The diversity is produced by alternative splicing of a single gene (Barbarese *et al*, 1977; Greenfield *et al.*, 1982). It was later shown that in fact the *MBP* gene lies within a larger gene region designated *golli-MBP* (Campagnoni *et al.*, 1993).

MBP are positively charged soluble proteins that are associated with the major dense line and are involved in maintaining the structure of compact myelin in both the central and peripheral nervous system. The different isoforms are expressed both spatially and temporally throughout development (Staugaitis *et al.*, 1990; Allinquant *et al.*, 1991).

In the *shiverer* mouse mutant all MBP isoforms are absent. The mice display a central nervous system deficit only, myelin is abnormal and the major dense line is absent. In the peripheral nervous system redundancy of function among the myelin proteins maintains myelin structure. For a more detailed discussion about the myelin basic protein family see section B1.3.

Peripheral Myelin Protein 22: Peripheral myelin protein 22 (PMP22) is an 18kDa protein that is glycosylated to a final molecular mass of 22kDa and is expressed predominantly in the peripheral nervous system. The carbohydrate entity contains the adhesion molecule L2/HNK-1 epitope. The expression of PMP-22 mirrors that of other myelin proteins during development and degeneration and subsequent regeneration. It is also expressed in a range of non-neural tissues where its function is unknown. Two alternate promoters mediate the widespread expression of PMP-22, one of which is specific to myelination. The role of PMP-22 in myelination is unknown although it shares properties typical of adhesion molecules. It is expressed concomitantly to P_0 and has been to shown to interact with this major structural protein of peripheral myelin. A mechanism is proposed where by the correct stoichiometry between the two proteins is necessary to maintain compact myelin. When this is disrupted through mutation of one of the components abnormal myelin may be formed (Pareek *et al.*, 1997; Notterpek *et al.*, 1999).

1.2 The Developmental Origins of the Schwann cell.

The subject of the following study focuses on myelination in the peripheral nervous system where this is accomplished by Schwann cells. The development, differentiation, and regulation of these cells are discussed below.

Following gastrulation the embryo comprises three distinct layers, the endoderm, mesoderm and ectoderm. Interaction between them initiates organogenesis and induces the ectodemal tissue to form the neural plate, neural folds rise and enclose to form the neural tube which will eventually give rise to the brain and spinal cord. The dorsal most portion of the neural tube becomes the neural crest cells. These cells differentiate into diverse cell types including Schwann cells (Le Douarin and Smith, 1988; Le Douarin *et al.*, 1991).

Crest cells destined to become Schwann cell precursors migrate through the anterior section of the somites that form along either side of the neural tube. This same pathway is taken by the first peripheral nerves as they emerge from the ventrolateral part of the

neural tube. In this way the Schwann cells and the neurons with which they must form an intimate relationship are present with the same spatial and temporal pattern enabling the initial interactions to occur (Rickman *et al.* 1985; Loring and Erickson, 1987).

Neural crest cells, destined to become Schwann cells that have not yet associated with neurons have been difficult to identify, although it has been shown that a protein more usually associated with mature Schwann cells can be used as a cell marker. A basal level of expression of the myelin protein, protein zero (P_O) is found on both precursor Schwann cells (Lee *et al.*, 1997) and a subpopulation of neural crest cells that are thought to have recently entered the Schwann cell lineage (Bhattacharyya *et al.*, 1991; Lee *et al.*, 1997). Subsequent differentiation of Schwann cells into the myelinating or non-myelinating form are accompanied by either an up-regulation or down-regulation of this basal level (Lee *et al.*, 1997).

Schwann cell precursors are defined by their interaction with embryonic neurons. By embryonic day 13 to 14, (E13/14) in the rat, nerves begin to extend into the hindlimb (Reynolds *et al.*, 1991). Around this time E14/15 (E12 and E13 in the mouse) precursors are a distinct cell type. *In vitro,* Schwann cell precursors are flattened and have extensive cell-cell contacts. In comparison, immature Schwann cells are bi- or tri-polar in appearance. Additionally, precursors lack the expression of the cytoplasmic dimeric calcium binding protein S100 that is characteristic of mature Schwann cells (Jessen *et al.*, 1994). The expression of S-100 in the nervous system is restricted to glial cells, astrocytes of the CNS and Schwann cells of the PNS (Brockes *et al.*, 1979).
The two populations of cells show distinct differences in their survival ability. When isolated from rat nerves at E14 the precursor cells undergo apoptosis within 20 hours. Cells isolated from neonatal rat nerves under the same conditions in contrast survive. (Jessen *et al.*, 1994; Dong *et al.*, 1995; Gavrilovic *et al.*, 1995; Mirsky and Jessen, 1996). The switch from precursor to immature Schwann cell types has been shown to occur between E15 and E17. The cells identified at E17 possess all the characteristics used to describe Schwann cells, survival, S100 expression and morphology (Jessen and Mirsky, 1997).

1.2.1 Survival factors from Schwann cells and Neurons

The death of isolated precursor Schwann cells indicated the absence of some factor necessary for their survival. An obvious difference between the two was the absence of neurons *in vitro*. It was shown that the death of Schwann cell precursors could be rescued by the presence of dorsal root ganglion (DRG) neurons. Media conditioned by DRG neurons was also sufficient to promote survival (Jessen *et al.*, 1994).

The axonally derived survival signal has been identified as β-neuregulin (also known as NDF and heregulin). The neuregulins are generated from a single gene and are present in both soluble and membrane bound forms (Pinkas-Kramarski *et al.*, 1994) that bind to members of the ErbB family of tyrosine kinases. They have been found to be involved in the growth and differentiation of several different cell types including epithelial, glial and muscle cells (Meyer and Birchmeier, 1995).

β-neuregulin is seen to support the survival of Schwann cell precursors, and their *in vitro* differentiation to immature Schwann cells with a time frame similar to that observed *in vivo* (Jessen *et al.*, 1994; Dong *et al.*, 1995). Additionally it is mitogenic for the immature Schwann cells, as their proliferation in response to axonal signals attempts to match the numbers of the two (Lemke, 1996; Jessen and Mirsky., 1999). These effects are lost when the cultures are incubated with a hybrid protein that incorporates the extracellular domain of the high affinity neuregulin receptor ErbB4 (Dong *et al.*, 1995).

Neural crest and Schwann cell precursors have been shown to express neuregulin receptors (Jin *et al.*, 1993; Dong *et al.*, 1995) and antibody blocking experiments have further corroborated the role of this molecule. The stimulation of immature Schwann cell proliferation brought on by incubation with axonal membranes was seen to be reduced 10 fold when either neuronal membranes were pre-incubated with anti-neuregulin or Schwann cells pre-incubated with anti-ErbB2 antibodies (Morrissey *et al.*, 1995).

Transgenic mice in which the neuregulin gene has been inactivated demonstrate the importance of this axonal signal. While heterozygous animals are healthy and survive, homozygous mutant embryos die during early embryogenesis. They show disruption of the development of the heart, with irregular heartbeat and malformations of the heart muscle. Schwann cell precursors fail to develop normally, as do the cranial ganglia

(Meyer and Birchmeier, 1995). The loss of this axonal signal has grave implications upon the survival of the Schwann cells and other critical areas of development.

A reciprocal dependence of axons on Schwann cell signals is inferred by the production of transgenic mice in which the neuregulin receptor ErbB3 is knocked out. Homozygous ErbB3$^{-/-}$ mice lack Schwann cells and their precursors, in addition the majority of sensory and motoneurons are lost. At E12 the numbers of sensory neurons present are normal, but by E14 they are reduced by 80%, motoneurons are lost at E18 (Riethmacher *et al.*, 1997). The death of these neuronal populations is thought to be due to the loss of a survival factor that would normally have been secreted by the absent Schwann cells. Possible candidate molecules include the glial cell line derived neurotrophic factor (GDNF). This is found in both precursor and immature Schwann cells and is a potent mitogen for motoneurons both *in vivo* and *in vitro,* mice lacking GDNF lose around 20% of their motoneurons by birth (Henderson, 1996; Moore *et al.*, 1996). GDNF however cannot account for all effects seen in the ErbB3-/- mouse as at the time of sensory neuron death in this mouse only a few of the sensory DRG neurons express the GDNF receptor. Since mice lacking Schwann cells suffer more extensively than those in which single growth factors have been deleted it is likely that a range of growth factors are involved in the regulation of axons (Molliver *et al.*, 1997).

1.2.2 Transcription Factor Regulation

A number of transcription factors have been implicated in the development of Schwann cells. The suppressed cAMP inducible POU domain protein (SCIP, also known as Oct-6) is expressed in Schwann cell precursors from E16, upregulated by axonal contact and is then downregulated as myelination proceeds (Scherer *et al.*, 1994; Zwart, 1996). Heterozygous mice in which this gene was disrupted with the *lacZ* gene show β-galactosidase activity in regions of SCIP expression, corroborating the expression pattern described previously. However the homozygous deletion proved more severe with the majority of pups dying soon after birth (Jaegle *et al.*, 1996). Those that did survive showed small and occasional tremors, suggesting deficiencies within the nervous system. Immunohistochemistry identified normal nerves in these animals at E18, yet at

postnatal day 8 (P8) their nerves possessed less myelin than wild type, although Schwann cells had formed the usual 1:1 relationship with the axons (Bermingham *et al.*, 1996; Jaegler *et al.* 1996). By P14, although levels were still reduced they had begun to rise, and at adulthood homozygous animals presented with normal myelination of the peripheral nerves (Jaegler *et al.*, 1996). This was interpreted to describe a role for SCIP in the regulation of genes involved in the transition from promyelinating to myelinating Schwann cells, but not in the terminal differentiation of the cells. Its loss causes a delay in the transitional step. If this can be overcome, as in the small number of homozygous animals that survive beyond birth, then normal myelination can follow.

This delayed effect on myelination seen in the SCIP knockout mouse contrasts with a mouse system in which ΔSCIP was expressed under the regulation of the P_O promoter. This is a dominant negative protein with respect to SCIP and was intended to inactivate endogenous SCIP in cells that had been induced to myelinate (Blanchard *et al.*, 1996). Unexpectedly it was seen that myelination was not blocked but induced prematurely and excessively (Weinstein *et al.*, 1995). This experiment was designed to occur in cells that had been stimulated to myelinate, inactivating the SCIP at a time when it is being downregulated. This may explain the conflicting results. The discrepancy may be due to differential roles for SCIP in early and late Schwann cell development (Weinstein *et al.*, 1995; Mirsky and Jessen, 1996).

Krox 20 is a zinc finger protein that is activated in Schwann cells before the onset of myelination and continues to be expressed throughout adult life (Topilko *et al.*, 1994). Its involvement in myelination is demonstrated by the inhibition of development of mature myelin in its absence. Schwann cells associate with individual axons and turn 1.5 times around the fibre. Early markers of myelin are present, such as S100 and myelin associated glycoprotein (MAG). The myelin proteins, myelin basic protein (MBP) and P_O, associated with mature myelin are reduced. Homozygous null alleles produced by insertion of the *lacZ* gene within the mouse Krox 20 gene show evidence of peripheral nervous system deficit with tremors that begin at 10-15 days after birth (Schneider-Maunoury *et al.*, 1993). Sciatic nerves from homozygous P15 animals revealed a total lack of myelin, whilst heterozygotes displayed a normal myelinated phenotype. The

formation of an association between Schwann cell and neuron and the absence of myelin debris suggests that Krox 20 is affecting the Schwann cell before compaction occurs but is not necessary for the initial interaction of the two cell types (Topilko et al, 1994).
The involvement of these two transcription factors at the same stage of development, from the pro-myelinating Schwann cell to the myelinating phenotype lead to an investigation into the interaction of the two. In P12 Krox 20 $^{-/-}$ mice SCIP levels are elevated, they have not declined as in the wild type mouse. This is accompanied by an increase in Schwann cell proliferation and apoptosis typical of SCIP+ve cells (Zorick *et al.*, 1999). It has also been shown that Krox 20 is able to cause the transactivation of P_O expression, a myelin protein the upregulation of which usually accompanies the decline in SCIP levels. This suggests that the two interact to allow the smooth transition between states of differentiation.

1.2.3 Autocrine Survival Mechanisms

Complex interactions between Schwann cells and neurons during development have been described that are crucial for the survival of both.
In the adult animal, however, the dependence of Schwann cell viability on axonal input is reduced. Mature Schwann cells possess an autocrine support loop and no longer depend upon axons for their survival. The importance of this distinction between the mature and precursor Schwann cells types is seen clearly following mature nerve injury when axons are seen to degenerate without significant loss in Schwann cell numbers (Trachtenberg and Thompson, 1996). If a loss of axonal signal were to result in the death of the surrounding Schwann cells axonal regeneration would be severely impaired as Schwann cells have been shown to provide a permissive substrate over which regenerating axons can grow (Hall, 1986; Fawcett and Keynes, 1990; Nadim *et al.*, 1990).

The autocrine loop is active from E18 and is seen in postnatal nerves, however it is absent in the Schwann cell precursors that require axonal signals for survival. Meier *et al.* (1999) investigated a range of different growth factors for their ability to support the survival of Schwann cells plated at very low density. At such low density autocrine

growth factors secreted by Schwann cells would be present in concentrations that are too low to be effective. Conditioned media from confluent cultures of Schwann cells, isolated from 7day old rat nerve, was sufficient to rescue the low density cells, preventing apoptosis. The effect of the conditioned media was replicated by the addition of platelet derived growth factor BB (PDGF-BB), insulin like growth factor (IGF-1 and IGF-2) and neurotrophin 3 (NT-3) specifically. Blocking antibodies against each of these growth factors inhibited their ability to prevent cell death. These three elements act synergistically to mimic the effect of the conditioned media.

This series of experiments was carried out over a period of two days in culture, long term cell survival was seen to be poor. When the experiments were repeated in cultures in which the Schwann cells were plated on laminin, a component of the Schwann cell basal lamina, survival times were increased. It is therefore likely that further signals are involved.

1.2.4 Summary

The development of the Schwann cell is controlled at many levels by axonal signals and the cell's own intrinsic mechanisms. Axonal input is important in the support of precursor Schwann cells and in stimulating the proliferation of immature Schwann cells. Coordinated regulation by transcription factors facilitates the progression then from promyelinating immature Schwann cells to the myelin forming phenotype. Throughout this the immature Schwann cells have developed an internal autocrine regulatory system that affords them independence from axonal signals for their survival.

1.3 Formation of the Myelin Sheath

As the immature Schwann cells become associated with axons, the properties of the neuron they segregate with determines their subsequent phenotype - namely myelin forming or non-myelin forming Schwann cells. The neuronal nature of this signal was demonstrated by a series of experiments by Aguayo *et al.* (1976) in which a graft from the sympathetic nerve that contains non-myelin forming Schwann cells, was introduced into a site of myelinated nerve. Following degeneration of the donor axonal fibres the

remaining Schwann cells were able to myelinate the host fibres, indicating that the ability to myelinate is intrinsic to all Schwann cells and that axonal phenotype determines the fate of the Schwann cell. The identity of the neuronal signal remains unknown.

1.3.1 Elaboration of Myelin

That the myelin sheath was a product of the extension of the Schwann cell plasmalemma was first realised by Geren in 1954. The study of electron micrographs of peripheral nerve identified Schwann cell elongation and spiraling around the axon, and as the spiral elongated the membranes within it extruded their cytoplasm and became compacted (Geren, 1954).

Once Schwann cell proliferation has ceased and the Schwann cell has identified with a single axon, it elongates along the axon defining the edges of the future internode. This is a positive association as competition between Schwann cells for access to the axon can result in less strongly attached Schwann cells falling off the neuron. Regular spacing of Schwann cell nuclei can be seen before the onset of myelination, and Schwann cell length does not increase as myelination proceeds.

During the initial stages of the myelin formation the Schwann cell nucleus is seen to circumnavigate the axon, this phenomenon slows as the sheath matures. Electron micrograph studies have shown that it is the inner lip of the myelin membrane that is traversing around the axon, with newly formed layers tucking under the previously deposited lamina (Raine, 1984; Webster and Favilla, 1984). The nuclear movement seen in the early stages occurs before a fully formed basal lamina is present and motion of the inner folds drags the sheath and Schwann cell nucleus with it. Once the basal lamina matures it anchors the external aspect of the myelin and nuclear movement ceases (Bunge *et al.*, 1989). The course of myelin formation can occur in either direction and independent Schwann cells associated with the same axon have been seen to spiral in opposing directions.

1.3.2 Basal Lamina

Associated with the myelin sheath are a number of layers of extra cellular material. As the myelin membrane ensheaths the axon, so it is ensheathed at the abaxonal surface by a basal lamina secreted by the Schwann cell. This in turn is surrounded by a collagen matrix (Peters *et al.*, 1976). The correct secretion of a basal lamina is essential for myelin formation. The Schwann cell basal lamina is composed of collagen types I, III, IV and V, laminin, entactin and heparin sulfate proteoglycan, HSPG (Bunge, 1986). The basal lamina is acquired by all Schwann cells early in development, as migration ceases and Schwann cells begin to segregate with individual axons (Webster, 1975).

It has been shown that axonal contact is necessary for the formation of the basal lamina (Bunge *et al.*, 1982). Schwann cells express laminin at all times in development, and when cultured in the absence of neuronal contact it is distributed in a punctate pattern. With the introduction of neurons, laminin expression becomes confluent throughout the basal lamina (Cornbrookes *et al.*, 1983). Another limiting component appears to be collagen type IV, in the absence of neurons the secretion of this protein is greatly reduced (Carey *et al.*, 1983).

In vitro tissue culture techniques have allowed analysis of the molecules involved in the promotion of basal lamina formation. In addition to neuronal contact, ascorbic acid and an unknown component of serum are essential for correct basal lamina production. Schwann cells cultured in a defined media in the absence of these factors are unable to produce a basal lamina and myelin. If this media is exchanged for a myelin permissive media that contains serum and ascorbic acid, a basal lamina is secreted and the Schwann cells will myelinate (Eldridge *et al.,* 1987).

Two lines of evidence demonstrate that the ascorbic acid affects myelination indirectly through the production of the basal lamina. In the central nervous system oligodendrocytes produce myelin in the absence of a basal lamina (Peters *et al.*, 1976), and cultures of oligodendrocytes do not need ascorbic acid to produce myelin, therefore this is not a requirement for myelin formation. Moreover ascorbic acid and serum can be replaced by the addition of exogenous basal lamina, but not other extra cellular matrices (Eldridge *et al.*, 1989). This indicates that a functional basal lamina is required before myelination can proceed.

Ascorbic acid is required for the hydroxylation of proline and lysine residues in type IV collagen (Wang *et al.*, 1989). This is necessary for the triple helical association of collagen chains and leads to increased secretion of triple helical type IV collagen (Eldridge *et al.*, 1987; Wang *et al.*, 1989). This is then assembled into a regular network that provides a mechanical stability to the matrix (Timpl *et al.*, 1981).

When normal secretion of the basal lamina is prevented, successive abnormalities of myelination are observed. Schwann cells associated with "guy roping" axons have no contact with the tissue culture dish substratum and are unable to form a basal lamina or myelin, however if they are forced to the surface of the dish, myelination can be promoted (Bunge *et al.*, 1989). A second surface is required by the Schwann cell, possibly to increase local concentrations of individual components. This polarisation leads to the reorganisation and morphological changes that accompany myelination, establishing plasmalemma domains. For example laminin receptors are expressed on the abaxonal surface of the Schwann cell, while those responsible for axonal contact will be localised to the adaxonal surface (Bunge *et al.*, 1986).

1.3.3 Non-myelinating Schwann cells

The molecular nature of the changes that accompany myelination have been extensively studied. This has elucidated the differences between the two Schwann cell phenotypes and determined important molecular changes that occur following injury.

Non-myelin forming cells may enclose a number of axons within a furrow of cytoplasm, ensheathing them within their membrane, yet the membranes do not show the same compact nature as myelin. The two Schwann cell types express distinct sets of surface membrane antigens and proteins, whilst they possess many of the same lipids.

Adult non-myelin forming Schwann cells have been shown to express the GFAP intermediate filament protein, the cell surface proteins A5E3 and 217c(Ran-2), and the cell adhesion molecules N-CAM and L1/Ng-CAM (Yen and Fields, 1981; Jessen *et al.*, 1984; Jessen and Mirsky, 1984; Mirsky and Jessen, 1984; Mirsky *et al.*, 1986; Martini and Schachner, 1986; Daniloff *et al.*, 1986). Each of these is not present or is found at much lower levels in myelin forming Schwann cells. During development the number of immature Schwann cells expressing A5E3 and 217c(Ran-1) increases from 75% of cells

at E15 to nearly all cells at birth. GFAP, first detectable at E18 is expressed in 50% of Schwann cells at birth. As myelination proceeds as indicated by the levels of myelin proteins present, these markers are lost in the majority of cells, and retained only in non-myelin forming Schwann cells, indicating the similarity between embryonic Schwann cells with the adult non-myelinating phenotype (Jessen *et al.*, 1990).

The loss of axonal contact has little effect on the expression patterns of non-myelin forming Schwann cells (Jessen *et al.*, 1990). Neural cell adhesion molecule, N-CAM is expressed on all neonatal Schwann cells and concurrent with myelination its expression is decreased in the myelin forming Schwann cells. However, within 24 hours of loss of axonal contact these cells re-express N-CAM (Jessen *et al.*, 1997), as well as other markers of non-myelin forming Schwann cells A5E3, 217c(ran-1) and GFAP.

1.3.4 Myelin Forming Schwann cells – the Expression of Myelin Proteins

Once myelination has begun, as the expression of proteins associated with neonatal and non-myelin forming Schwann cells decline, a concomitant upregulation of a number of myelin proteins occurs in a temporal and spatial pattern. Immunohistochemical and *in situ* studies have been used to describe the appearance of structurally important myelin proteins.

During development, as immature Schwann cells associate with myelin promoting axons a basal lamina is formed and the Schwann cells begin to myelinate at birth, reaching a peak at postnatal day P7-14. The major myelin proteins peripheral myelin protein 22 (PMP-22) and protein zero (P_O) are detectable at birth. A rapid increase in expression reaches half maximal levels at P3 and a maximum by P7, levels then decline slightly to stable adult levels. The expression of the remaining structural proteins of the myelin basic protein, MBP family occurs with a slight delay, first detectable at P3 with a maximum expression at P14. There is no significant decline in adulthood (Kuhn *et al.*, 1993; Notterpek *et al.*, 1999). The myelin associated glycoprotein (MAG) is readily detected at P1and is near maximal by P4, this is in line with its proposed role in the initiation of early axon Schwann cell interactions (review Martini and Schachner, 1997; Yin *et al.*, 1998).

The expression of myelin proteins is dependent upon axonal signals (Lemke and Chao, 1988). The withdrawal of axons from tissue culture or following nerve crush or transection results in the downregulation of myelin proteins, expression is recovered once axonal regeneration has taken place. Within three days of nerve injury there is a rapid decline in the levels of PMP-22, P_0 and MBP in the distal nerve segment. The time course of the reinduction of the three proteins is tightly correlated to axonal regeneration (Kuhn *et al.*, 1993). Bolin and Shooter (1993) have shown that at least one component of the axonal signals involved in this process is soluble. In neuronal co-cultures in which axons and Schwann cells are unable to make direct contact induction of a myelin associated transcription factor, SCIP and myelin protein P_0 is seen, indicating the diffusible nature of the signal.

Despite the close correlation in the expression of PMP-22 and P_0, distinct differences exist in their regulation. In non-myelin forming Schwann cells stimulated with forskolin (an activator of cAMP), low levels of P_0 and PMP-22 expression are seen. cAMP has been implicated in mediating the signals between axons and Schwann cells (Lemke and Chao, 1988; Morgan *et al.*, 1991). Whilst P_0 was inserted into the plasma membrane, PMP-22 expression was restricted to the Golgi compartment. The introduction of neurons was able to promote the translocation of PMP-22 to the plasma membrane in the presence of a basal lamina (Pareek *et al.*, 1997).

1.3.5 The Effects of Demyelination

In the peripheral nervous system successful regeneration of function following nerve injury is possible. The basal lamina deposited by the Schwann cells remains after axonal loss and degradation of the myelin sheath. This environment is permissive for the regeneration of both PNS and CNS axons.

Axonal crush is followed by a defined sequence of histopathological reactions termed Wallerian degeneration. This is charactersied by a loss of axons and myelin breakdown; changes in the permeability of blood vessels; Schwann cell proliferation; invasion of the trauma site by phagocytes; and the phagocytosis of myelin debris by Schwann cells and macrophages (for review Sunderland, 1978). The loss of axonal signals causes the

downregulation of the myelin proteins and the return of expression of proteins characteristic of non-myelin forming Schwann cells (section 1.3.4). The existing myelin sheath is broken into myelin fragments and ovoids. The sum of the data available suggests that the Schwann cell itself initiates the degradation process and that the appearance of macrophages following this then takes over the majority of this process. Myelin degradation is then quickly and efficiently completed (Goodrum and Bouldin, 1996).

Within hours of the nerve crush injury the *de novo* lipid synthesis is reduced by 50% in the Schwann cell and by 3 days post crush has all but ceased entirely. In contrast to the immediate reaction of lipid synthesis, lipid degradation does not begin until a week after the initial trauma, but subsequently proceeds rapidly. This timing corresponds to the appearance of macrophages at the injury site. The lipids are broken down into droplets that are then localised to the macrophages.

Myelination is accompanied by a massive increase in the production of plasmalemma, a major part of which is accounted for by lipids. Radiolabelled studies have shown that the cholesterol components of an existing myelin sheath that are labelled before breakdown begins are subsequently present in newly synthesised myelin sheaths following regeneration (Rawlins *et al.*, 1970; 1972). This recycling of the cholesterol is consistent with the observation that the overall total amount of cholesterol does not change during degeneration (Goodrum, 1991). Further investigation has revealed that while all phospholipids are degraded, approximately half of all fatty acids are also reincorporated into new myelin membranes (Goodrum *et al.*, 1995). For review see Goodrum and Bouldin, 1996.

Following myelin breakdown some of the Schwann cells proliferate, as is the case during the developmental proliferation of Schwann cells, this is mediated by axonal contact and membrane interaction between the two is necessary for the stimulatory effect of neurons. If the axons and Schwann cells are separated by a permeable membrane, the stimulation to divide is lost (Salzer and Bunge, 1980; Salzer *et al.*, 1980a, 1980b). As axonal regeneration occurs through the site of injury connections are made with the appropriate target and myelination and upregulation of myelin proteins begins again. The resulting myelin sheath tends to have slightly shorter internodes but

normal cytochemical properties, and the axonal signal is propagated at near normal conduction velocities (Cragg and Thomas, 1964; Rasminsky, 1978; Weiner *et al.*, 1980).

1.3.5.1 Trophic Factors

The signals that circulate during different stages of differentiation of the Schwann cell and axon relationship are also subject to change following nerve injury. For example the nerve growth factor (NGF), that promotes the survival of peripheral sensory and sympathetic neurons (Levi-Montalcini, 1987) and cholinergic neurons of the forebrain (Williams *et al.*, 1986) are increased following nerve injury at the distal site. Levels decline once regeneration is complete (Heumann *et al.*, 1987). Since Schwann cells posses high levels of a receptor for NGF following loss of axonal contact during injury, it may be that in binding the trophic factor they are providing a localised source on their surface, over which regenerating neurons migrate (Johnson *et al.*, 1988). As myelination resumes, NGF expression falls, as does the level of the receptor on the Schwann cell surface.

Ciliary neurotrophic factor (CNTF) is upregulated by Schwann cells following axonal contact (Dobrea *et al.*, 1992; Friedman *et al.*, 1992) as is brain derived neurotrophic factor (BDNF, Sendtner *et al.*, 1992). These molecules support different neuronal populations than NGF (Reynolds and Woolf 1993).

1.4 Peripheral Myelin Protein 22

During the late 1980's the mechanisms of nerve degeneration resulting from injury to the peripheral nervous system had become well recognised, yet much of the molecular basis of this process was unknown. The expression of the myelin proteins MBP and P_0 had been shown to be regulated during the loss of axonal contact and an attempt was made to describe other gene products that were regulated during regeneration. Peripheral myelin protein-22 (PMP-22) was identified independently by two groups from rat sciatic nerve cDNA libraries as being differentially regulated following nerve crush.

Welcher *et al.* (1991) isolated SR13. This described a 1.8kb mRNA transcript that was rapidly repressed to less than 5% of normal values in the distal nerve segment 3-14 days

after nerve crush injury. By 40 days post crush, levels had recovered and were approaching those of control nerves. When the distal segment was prevented from regenerating transcript expression remained low. Spreyer *et al* (1991) identified a transcript designated CD25 with a similar expression pattern during degeneration and regeneration of nerve crush. The connection between expression and neuronal contact was confirmed in a series of experiments where nerve sections that had been prevented from regenerating were reconnected. Within one week nerves were seen to migrate into the distal stump, this was followed a week later by the expression of the CD25 transcript.

Both groups described a 1.8kb transcript that encoded for a 160 amino acid protein with a predicted molecular mass of 18kDa. The addition of a carbohydrate moiety at a consensus N-linked glycosylation site at Asn41 results in a final protein of 22kDa. Hydrophobicity plots and secondary structure predicts four putative transmembrane spanning domains. The N-terminus contains a putative signal peptide with a predicted cleavage site between Ser26 and Gly27, however cleavage is not seen. In addition phosphorylation sites are found at Ser57 and Thr118.

Despite the very high homology of rat SR13/CD25 with the murine Gas-3 growth arrest specific protein it was thought to be a potential myelin protein since the described regulation of expression following nerve crush reflected that of other myelin proteins (Lemke and Chao, 1988; Trapp *et al.*, 1988). Levels of SR13/CD25 increase developmentally rising from birth to a peak by postnatal day 21 (Snipes *et al.*, 1992). RNA antisense probes and myelin specific antibodies localised the expression to S100 immunoreactive Schwann cells of peripheral nerves (Spreyer *et al.*, 1991; Welcher *et al.*, 1991; Snipes *et al.*, 1992). Given these characteristics, the protein was renamed peripheral myelin protein 22 (Snipes *et al.*, 1992).

The coding sequence and 3' untranslated region of SR13 and CD25 have been shown to be identical, however discrepancies occur in the sequence of the 5' untranslated region. The regulatory region of SR13 is identical to that of the *GAS-3* gene, yet distinct from that of CD25. Investigation of the *PMP-22* gene elucidated the gene structure. PMP-22 is coded for by four exons, designated exons 2-5. Exon 2 codes for the first

transmembrane spanning domain. Exon 3 carries the extracellular loop that includes the N-linked glycosylation site. The second transmembrane spanning domain and half of the third is encoded for by exon 4. Exon 5 codes the remainder of the third membrane spanning domain, the fourth transmembrane domain, and the 3' untranslated region. Before these coding regions lies two non-translated exons 1a and 1b that are responsible for the CD25 and SR13 transcripts respectively. Each of these in turn is regulated by the alternative promoters P1 and P2 (Suter *et al.*, 1994).

The alternative transcripts were shown to have disparate expression patterns. During development the exon 1a containing (CD25) transcript can be seen to rise from birth to peak at P14, initially the transcript amounts to only 30% of the total PMP-22 mRNA present, it rises to 80% of the total in the postnatal period corresponding to myelination. Following nerve injury its expression becomes undetectable 3 days post-crush. By the second week levels begin to recover, and at 21 days after the injury it is the major PMP-22 transcript.

In comparison, the exon 1b containing transcript levels rise from birth to peak at postnatal day 4 and then decreases until postnatal day 21. Nerve injury results in a rapid downregulation in the first 3-7days and control levels have returned by four weeks. This does not parallel the formation of myelin and the expression patterns of other myelin proteins (Bosse *et al.*, 1994; Suter *et al.*, 1994).

The gene structure presents a gene whose expression is controlled by two alternate promoters. P1 and exon 1a containing transcripts are involved in the formation and maintenance of peripheral myelin. The P2 promoter and exon 1b transcripts are more strongly expressed in non-neural sites and may have a separate function to that implicated in myelination.

1.4.1 Non-neural Expression of PMP-22

Manfioletti *et al.* (1990) reported the presence of the growth arrest specific Gas-3 in NIH3T3 fibroblasts. Further investigation found detectable levels in other tissues such as the lung and colon. Northern blot studies and the use of RT-PCR have identified a number of non-neural sites of expression for both transcripts of PMP-22.

In the adult mouse, weak extra-neural expression of PMP-22 is seen in the lung, liver colon, skeletal muscle, testes. In the brain PMP-22 mRNA but not protein is found

(Spreyer *et al.*, 1991; Welcher *et al.*, 1991; Snipes *et al.*, 1992). The central nervous system expression has been shown to be restricted to the motor neurons of the spinal cord, ventral horn and the cranial nerve motor nuclei (Parmantier *et al.*, 1995, 1997).
Bosse *et al.* (1994) used RT-PCR to further distinguish between the nature of the PMP-22 transcripts. The exon 1a containing transcript was found to be near or below detection levels in all tissues except the peripheral nerve, whilst exon 1b transcripts were expressed in the lung, skeletal muscle and the colon. In contrast a later report has found expression of both transcripts in the heart, lung, skeletal muscle, testis, stomach and tongue (Van der Weetering *et al.*, 1999).
This diverse expression of PMP-22 and the existence of alternative promoters and non-translated exons suggest a dual function for the PMP-22 protein.

1.4.2 The PMP-22 Gene Family

A number of hydrophobic proteins have been described that share structural similarities, transmembrane spanning domains and glycosylation sites with PMP-22. Recently a group of these have been shown to belong to the same gene family.
This subgroup of membrane spanning proteins consists of PMP-22, the epithelial membrane proteins EMP-1, EMP-2 and EMP-3, and a more distant member the lens epithelial membrane protein MP20 (Church and Wang, 1993; Taylor *et al.*, 1995; Marvin *et al.* 1995; Taylor and Suter, 1996; Bolin *et al.*, 1997). Each protein shares amino acid sequence homology with PMP-22, with the highest degree corresponding to the four transmembrane spanning domains, particularly that of the second transmembrane region. The first hydrophilic loop contains one or more sites for N-linked glycosylation sites, and in EMP-1 one of these is conserved with that of PMP-22 (Marvin *et al.*, 1995).
The gene encoding each has been shown to be independent from, but related to that of PMP-22 (Jetten and Suter, 2000).

The epithelial membrane proteins EMP-1, EMP-2 and EMP-3 all show extensive expression in non-neural tissues, many sites of which are shared by PMP-22. However the regulation of these proteins in the nervous system is distinct from that of PMP-22.
A study into the expression of EMP-1 during nerve injury has shown that initially EMP-1 levels are lower than those of PMP-22 in sciatic nerve. Following trauma the levels are inversely correlated, EMP-1 levels rise whilst PMP-22 levels are reduced (Taylor *et al.*, 1995). This contrasting expression pattern is also seen following forskolin stimulation of Schwann cell cultures and serum starvation induced growth arrest. As PMP-22 levels increase those of EMP-1 decrease (Taylor *et al.*, 1995; Ben-Porath and Benvenisty, 1996). In support of this increase in expression during proliferative conditions *in vitro* the greatest expression of EMP-1 *in vivo* is seen in the proliferation and differentiation zones of the outer gastric gland and the mature epithelia of the gastric pit.
Bolin *et al.* (1997) studied the expression of EMP-3/HNMP-1 during the development of peripheral nerves. During embryogeneis at E12, the spinal cord nerve fibres display EMP-3 expression, and by E16 it is seen in the cell bodies of motoneurons. In the adult sciatic nerve EMP-3 is barely detectable but localised to the axon. After nerve crush, EMP-3 is rapidly induced distal to the injury. One week post crush, the EMP-3 expression is no longer axonal, but is localised to regions of Schwann cell proliferation. Three weeks post crush, and the axon associated expression has returned. Expression continues after functional recovery of the injury, but with a time scale that suggests an active role in myelination.

The membrane protein MP20 is a major component of the eyes lens and shares a lesser degree of sequence similarity with PMP-22 (25-29%), despite this, the major regions of conservation across the family are maintained (Taylor and Suter, 1996). It co-localises with connexin 46 at the junctional plaques of the lens. Expression is high in differentiating and mature lens cells, but it is absent from the proliferating cells (Tenbroek *et al.*, 1992; Kumar *et al.*, 1993).

Despite other non-neural sites of expression, mutations in the *PMP-22* gene affect only the peripheral nervous system. It has been suggested that in these non-neural tissues, other members of the PMP-22 family could substitute for PMP-22 therefore the phenotype is not extended to these sites. It has been shown, however, that although they show expression in a similar range of cell types, the regulation of alternative family members during proliferation and growth arrest is discrete and often conflicting to that of PMP-22.

1.5 Growth Arrest

The wide expression pattern of PMP-22 in non-neural tissues and its homology with the mouse growth arrest protein Gas-3 suggest it may be involved in cell cycle regulation. Supporting evidence is found in human and mouse disease caused by mutation of PMP-22, Carcot-Marie-Tooth disease and the mouse models *trembler* and *trembler-J.* These neuropathies are accompanied by abnormal Schwann cell proliferation.
Further investigations into the possible effects of PMP-22 on the cell cycle have indicated some involvement, although an exact role is still unknown.

A characteristic of the immature Schwann cell phenotype is that both proliferation and programmed cell death are found in their population. Cell death is most evident during the first two postnatal weeks and levels decline in the adult (Grinspan *et al.*, 1996; Syroid *et al.*, 1996; Trachtenberg and Thompson, 1996). It is thought this period of apoptosis matches the number of Schwann cells to axons and in this way the correct balance for myelination and functional neuronal activity is achieved (Nakao *et al.*, 1997).
In the disease state associated with Charcot Marie Tooth disease, abnormal Schwann cell proliferation has been associated with a subsequent decrease in Schwann cell number. In addition, an increase in the number of cells undergoing programmed cell death is observed. It is thought that this is a result of the axonal loss seen during progression of the disease (Erdem *et al.* 1998).

1.5.1 Growth Arrest Specific Protein-3

Biochemical and genetic changes are involved throughout the cell cycle that are modulated by both positive and negative signals (Craig and Sager, 1985). A number of genes have been identified that are concerned specifically with the G_0G_1 state (Pepperkok *et al.*, 1988; Schneider *et al.*, 1988), the growth arrest specific gene *GAS-3* is one of six genes expressed at this time in NIH3T3 fibroblasts (Schneider *et al.*, 1988).

It has been shown that the *Gas-3* gene sequence is identical to that of *PMP-22* and includes the 5' untranslated region of the P2 exon 1b containing transcript (Manfioletti *et al.*, 1990; Bosse *et al.*, 1994).

1.5.2 Expression of PMP-22 in Growth Arrest

Suter *et al.* (1994) found that following growth arrest of rat embryonic fibroblasts, the increase in expression of PMP-22/Gas-3 was less dramatic than that observed in NIH3T3 fibroblasts. Studies showed that both the transcripts of PMP-22 were induced to similar levels, and the ratio between the two did not alter during growth arrest. This was corroborated by Zoidl *et al.* (1995) in rat Schwann cells. They were able to show by RT-PCR that following serum deprivation the levels of both PMP-22 transcripts increased. Serum induced re-entry into the cell cycle caused a further initial increase in both, that was more pronounced in the 1b transcript than the 1a. This then decreased to undetectable levels within 6 hours.

In contrast Bosse *et al.* (1994) found that the PMP-22 exon 1b containing transcript was exclusively upregulated in arrested rat meningeal fibroblasts, however no growth arrest-specific expression of either transcript was found in rat Schwann cells.

The over and under expression of PMP-22/Gas-3 in Schwann cells through retroviral mediated transfection gave further insight into possible mechanisms of action of PMP-22 in cell cycle control. The introduction of excess amounts of sense cDNA coding for PMP-22 caused the number of cells in the S phase of the cell cycle (undergoing DNA synthesis as described by BrdU staining) to decrease. The expression of an antisense construct, however, resulted in enhanced levels of BrdU incorporation. A delay of eight hours in the entry of resting Schwann cells into the S phase of the cell cycle was caused by the overexpression of PMP-22 (Zoidl *et al.*, 1995). This suggests that the site of

action of PMP-22 lies in the transition between G_0G_1 and the S phase, a process known to be under strict regulatory control (Pardee, 1989; Muller *et al.*, 1993).

1.5.3 Apoptosis and PMP-22/Gas 3 Expression

In NIH3T3 cells an overexpression of the wild type PMP-22 was seen to give rise to a distinct phenotype. More than 50% of cells became loosely attached to the substratum, they showed condensed nuclei and blebbing of the plasma membrane was observed (Fabretti *et al.*, 1995). This morphological appearance is characteristic of cells undergoing apoptosis (Kerr *et al.*, 1971; Wyllie *et al.*, 1980; Evan *et al.*, 1992). However no evidence for increased apoptotic activity was suggested by study of nuclear fragmentation using the TUNEL technique (Gravieli *et al.*, 1992).

Using the morphological criteria for programmed cell death described above, mutations in the PMP-22 gene were evaluated for the ability to induce apoptosis. All the mutations studied, including that responsible for the Tr-J mouse showed a normal phenotype.

Zoidl *et al.* (1997) also investigated the role of PMP-22 in NIH3T3 fibroblasts. Overexpression of the wild type protein was found to reduce the number of viable cells in culture by an average of 61.9% (+/- 23.8%). Overexpression of the cDNA for the trembler mutant form of the protein however caused only a 31.8% (+/- 14.2%) reduction in viable cells in comparison to controls. Cells were seen to be arrested at G_1 with reduced numbers in the S and G_2 phases, although all transfected cells were able to progress through the cell cycle. This indicates again that PMP-22 is involved in the progression of the cell through the transition between G_1 and S phase and that this is disrupted in the mutant mice.

In response to the observations of Fabbretti *et al.* (1995), the role of programmed cell death in the reduction of viable cells was investigated. In previous studies the overexpression of PMP-22 in Schwann cells did not give rise to any evidence of apoptotic activity (Zoidl *et al.*, 1995).

Assessment of apoptosis via nuclear staining and the TUNEL assay for DNA fragmentation indicated an increase in cell death by 4% with the overexpression of wild type PMP-22. The mutated protein, with the point mutation characteristic of the *trembler* mouse, caused no detectable apoptosis.

The degree of apoptosis that occurred in the two studies greatly differed, Fabbretti *et al* (1995) reported over 50% of cells showed evidence of cell death, whilst Zoidl *et al.* (1997) described apoptosis in only 4% of cells. The discrepancy may be due to the levels of overexpression of the protein or the method of detection.

It has been demonstrated that withdrawal from the cell cycle after growth factor deprivation increases the cellular sensitivity towards apoptosis (Raff, 1992; Hoffman and Liebermann, 1994). If PMP-22 expression is maintaining and regulating the growth arrest phase of the cell cycle it follows that a consequence of this may be increased apoptosis.

The lack of evidence for an apoptotic role for PMP-22 in Schwann cells may reflect an increased tolerance of the cell to PMP-22, necessary because of the strong upregulation of its expression during myelination. Alternatively it may represent observations that different cell types vary in their activation of the cell death program (Collins and Lopez Rivas, 1993; McConkey and Orrenius, 1994).

1.6 Disorders of the Peripheral Nervous System

The hereditary motor and sensory neuropathies (HMSN) comprise of a group of diverse disorders that affect the nervous system. The causative genetic defects in a number of the peripheral neuropathies have been determined with the aid of studies into mouse mutant models.

1.6.1 Charcot Marie Tooth Disease

First described in 1886 by Jean Marin Charcot and Pierre Marie (Charcot and Marie 1886) and Howard Henry Tooth (Tooth, 1886) the disease bears their names, Charcot Marie Tooth disease (CMT). From the age of onset at 12.2 years (+/- 7.3 years, Lupski *et al.*, 1991a), patients show progressive distal muscle weakness and atrophy that often begins in the feet and progress to the hands and arms. There is impairment of sensation and diminished tendon reflexes (Dyke *et al.*, 1993). A significant reduction in the nerve conduction velocity (NCV) is also found (Nicholson, 1991) and is thought to be due to the progressive loss of axons (Lupski *et al.*, 1991b). Nerve biopsies from CMT patients present with "onion bulbs" a sign of repeated demyelination followed by remyelination,

followed by remyelination, in addition large calibre axons are enclosed by abnormally thin sheaths (Suter and Snipes, 1995).

The clinical features of the disease can be diverse, and it can be categorised further. The most common form is Charcot Marie Tooth disease type 1 (CMT-1). The disease is characterised by a reduced NCV and repeated demyelination/remyelination cycles. Patients with CMT type 2 however have near normal NCV and the main pathological feature is the loss of large myelinated axons (Dyke *et al.*, 1993). A more severe form of the disease, known as Dejerine-Sottas disease (DSS) is also observed. Patients show marked hypermyelination of the peripheral nervous system and onion bulb formation (Suter and Snipes, 1995).

The genetic defect of CMT-1 in the majority of patients has been mapped to chromosome 17 (Ionascescu *et al.*, 1993), although a minority of the defects arise on chromosome 1 (Bird *et al.*, 1982). On this basis the disease was further subdivided as CMT type 1A and CMT type 1B respectively, the clinical symptoms of the two are indistinguishable (Dyke *et al.*, 1993).

An X-linked form of the disease CMT-X occurs in 10% of patients (Skre, 1974). Childhood onset, progressive distal weakness and sensory loss are more severe than in CMT-1. The reduction in NCV however is less than that of most CMT-1 patients (Suter and Snipes 1995).

1.6.2 Hereditary Neuropathy with Liability to Pressure Palsies

First described by Davies in 1954 hereditary neuropathy with liability to pressure palsies (HNPP) presents symptoms in adolescence. Patients show acute or recurrent transient muscle palsies that can be precipitated by trauma or pressure to peripheral nerves (Amato *et al.*, 1996; Windebank, 1993). The pathological characteristics are multiple focal thickenings of myelin that form sausage shaped enlargements along the axon fibre, known as tomacula. Some individuals show a reduction of the NCV and evidence for demyelination and remyelination more commonly associated with CMT but no onion bulb formation (Windebank, 1993).

1.6.3 Genetics of Disease

In 70-80% of CMT-1A sufferers, the mutation in CMT-1A is caused by a duplication of 1.5 megabases of chromosome 17 (Lupski *et al.*, 1991a; Raeymaekers *et al.*, 1991; Wise *et al.*, 1993). The duplicated region is flanked by repeated sequences 17-19kb in length. *De novo* duplications arise from the misalignment of these repeats during meiosis (Pentao *et al.*, 1992; Chance *et al.*, 1994), and are overwhelmingly of paternal origin (Palau *et al.*, 1993). The reciprocal deletion of this region is seen in patients with HNPP (Chance *et al.*, 1993).

1.6.4 Spontaneous Mouse Mutant Models of Disease

A number of different spontaneously occurring mouse mutants have been described that present with affected nervous systems. Two stand out as displaying parallel phenotypes to the human peripheral neuropathy diseases. The *trembler* (*Tr*) mouse and its allelic mutant *trembler J* (Tr^J) both show behaviour deficits as a result of abnormal myelin formation in the peripheral nerves.

1.6.4.1 The *Trembler* Mouse

The *Tr* mutation shows an autosomal dominant pattern of expression. The affected mice show tremor and transient seizures with no abnormality of the central nervous system. The morphology of the peripheral nervous system shows hypomyelination at an early stage. Evidence of demyelination can be seen, and the progressive nature of the phenotype is evident by the presence of onion bulb formation in older animals. Onion bulbs consist of a central thinly myelinated axon surrounded by supernumerary Schwann cell processes that have increased amounts of interstitial tissue (Suter and Snipes 1995). Despite the abnormalities, mice homozygous for the mutation are long lived (Henry and Sidman, 1988).

Many Schwann cells can be seen to be in a pro-myelin state, having formed a 1:1 relationship with the axon more typical of neonatal myelin formation. The initial axon Schwann cell interactions are maintained, whilst later stages of myelination are affected by the loss of PMP-22.

A great reduction in the number of myelinated fibres is seen, and those that are myelinated have sheaths that are thin relative to the calibre of the axon. The myelin is poorly compacted, particularly at the node of Ranvier, and the nodes may be wider than normal. The Schwann cells also contain cytoplasmic debris (for review see Suter and Snipes 1995). The hypomyelination is reflected in the expression of myelin proteins. The levels of mRNA for compact myelin proteins such as P_O and MBP are greatly reduced (Jacque *et al.*, 1983; Bascles *et al.*, 1992; Garbay and Bonnet, 1992), while MAG levels are unchanged (Inuzuka *et al.*, 1985).

In the wild type mouse Schwann cells cease proliferation after the first few postnatal weeks, in the *Tr* mouse, increased Schwann cell numbers are seen as proliferation continues throughout life (Perkins *et al.*, 1981a). A similar pattern of Schwann cell proliferation has been described in CMT and HNPP patients (Behse *et al.*, 1972; Perkins *et al.*, 1981a; Henry *et al.*,1983).

It has been suggested that the abnormal proliferation of Schwann cells in *Tr* mice may be due to stimulatory signals released during the repeated cycles of myelin breakdown (Salzer and Bunge, 1980; De Vries *et al.*, 1982; Koenig *et al.*, 1991). Tissue culture studies show that the abnormal proliferation rates of *Tr* Schwann cells are lost *in vitro* (White *et al.*, 1986; Do Thi *et al.*, 1993).

1.6.4.2 Trembler J

The semi-dominant mutation in the *trembler J* mouse arose spontaneously and independently from the *trembler* mouse (Henry *et al.*, 1983). It has been proposed to be allelic to *trembler* through linkage studies and the similarity of phenotype (Henry and Sidman, 1983).

Heterozygotes for the Tr^J mutation can be identified behaviourally by postnatal day 20. P20 to P25, they have a mild tremor with no obvious seizures, and a gait abnormality affecting the hind limbs progresses only slightly with age (Henry *et al.*, 1983; Robertson *et al.*, 1997). The severity of disease in heterozygous Tr^J animals is less than that of *Tr* heterozygotes, yet it is more severe in the homozygotes. Homozygous Tr^J mice are obvious by P5-P8, they are smaller animals and display severely dystonic, ineffective

movement and have difficulty in righting themselves. They do not survive beyond P17-P18 (Henry *et al.*, 1983).

The morphology of peripheral myelin in Tr^J mice is affected. At P14 some of the large diameter axons have no visible myelin, although by P16 most posses a thin layer. By 1 year of age nearly all axons have some myelin sheath, no evidence is seen of myelin breakdown and onion bulbs are absent (Suter and Snipes, 1995). The deficiency is greater in homozygous animals as is reflected by the severity of the phenotype. Immunolocalisation studies show reduced levels of MAG and P_O in golgi associated vesicles in some Schwann cells (Heath *et al.*, 1991), PMP-22 immunoreactivity is also seen to co-localise with lysosomal markers (Notterpek *et al.*, 1997).

More subtle abnormalities are seen in the relationship between Schwann cells and axons in the Tr^J mouse (Robertson *et al.*, 1997). These include the morphology of the terminal loop of myelin and the position of the Schwann cell body relative to the centre of the internode. No onion bulbs are observed of the type described in *Tr*, however abnormalities in the basal lamina of Tr^J Schwann cells are seen - layers of collapsed basement membrane possibly representing processes extended by Schwann cells that were later withdrawn.

1.6.5 Identifying the Site of the Mutation

The identification of the affected gene in the *trembler* and *trembler J* phenotype was preceded by a number of discoveries. In a series of experiments, Aguayo *et al.* (1977) demonstrated that the likely primary target of the defect was the Schwann cell. When *Tr* nerve grafts were introduced into a normal mouse host, the axons degenerated leaving the non-neural component. Subsequently abnormal myelin representative of the *Tr* phenotype was found at the site of the graft. In the reciprocal experiment, when normal Schwann cells attempt to remyelinate *Tr* axons the typical myelinated phenotype was observed. Schwann cells from sympathetic nerves of the *Tr* mouse, which are ordinarily non-myelinating and show no abnormality in the mutant mouse, were also grafted into the site of normally myelinated axons. Again the *trembler* phenotype was seen, suggesting that the defect exists in all Schwann cells (Perkins *et al.*, 1981b)

The chromosomal position of the *trembler* and *trembler J* mutation were shown to map to mouse chromosome 11, which has synteny with human chromosome 17 the site of the CMT duplication. In addition the gene for the peripheral myelin specific gene of PMP-22 has also been shown to reside on chromosome 11 (Suter *et al.*, 1992a). Analysis of the sequence of the PMP-22 gene in the mouse mutants discovered the presence of a point mutation in each that was thought to be the likely cause of each phenotype.

In the *Tr* mouse a guanine to adenine substitution results in a change from glycine to aspartic acid at amino acid 150. This introduces a charged amino acid into the putative fourth transmembrane spanning domain of the PMP-22 protein which may affect its structure and function (Suter *et al.*, 1992a). The Tr^J mutation has a thymine to cytosine alteration at nucleotide 190 that results in a leucine to proline substitution at amino acid position 16 in the first putative transmembrane spanning domain of the protein (Suter *et al.*, 1992b). The transmembrane spanning regions of the PMP-22 gene are the most strongly conserved, suggesting that they are important in PMP-22 function, and this is reflected in the phenotype resulting from their disruption. The mechanism of action of the mutation is unknown. The mutated protein may be unable to incorporate correctly into the plasmamembrane preventing the development of compact myelin. Alternatively the mutation may affect conformation changes to the protein structure interupting the normal interactions of the protein with itself or other proteins.

The mutations in Tr^J have been shown to alter the post-transcriptional regulation of the PMP-22 protein. Notterpek *et al.* (1997) described the upregulation of the lysosomal pathway in the mice. This lead to the hypothesis that the mutated form of the PMP-22 protein was successfully glycosylated and then incorporated into the plasma membrane where it is unstable. Autophagy and/or endocytosis is then upregulated in the Schwann cells to remove the myelin proteins. It has recently been shown that the mutated protein never reaches the plasma membrane but is arrested in the vesicles of the intermediate compartment.

A number of groups have shown that a minor portion of the wild type PMP-22 protein is targeted to the plasma membrane, while a second population is retained in the endoplasmic reticulum where it is rapidly turned over (Naef *et al.*, 1997; D'Urso *et al.*, 1998; Naef and Suter, 1999; Tobler *et al.*, 1999). Tobler *et al* (1999) and Naef and Suter

(1999) investigated the effects of different point mutations on the transport of the PMP-22 protein, including that of the *trembler-J* mutation. It was found that with all mutations found in the putative transmembrane spanning domains, regardless of the severity of disease seen as a consequence, no PMP-22 protein was found in the plasma membrane. The mutated proteins were found to remain in the endoplasmic reticulum or the vesicles of the intermediate compartment that are involved in transport from the endoplasmic reticulum to the golgi apparatus.

When the wild type and a mutated form of the PMP-22 were co-expressed, a reduced amount of the wild type was inserted into the membrane. A proportion remained in the intermediate compartment with the mutated protein. This suggested interaction between the two and this was confirmed in Western blot studies that identified potential dimer formation by the wild type PMP-22 protein (Tobler *et al.*, 1999).

What this data suggests is that point mutations result in the altered distribution of PMP-22. When the gene is duplicated or deleted the balance of myelin proteins may disturb the physiology of Schwann cells. Changes in the stoichiometry of the myelin proteins could disrupt the interactions of PMP-22 with itself or with P_0 (D'urso *et al.*, 1999) and so affect the structure of compact myelin that typifies mutations of PMP-22.

1.6.6 PMP-22 as the Primary Defect in CMT and HNPP

Once PMP-22 had been identified as the site of the mutation in the mouse mutant models of peripheral neuropathy, it became a candidate for the gene responsible for human disease. Analysis showed that the human PMP-22 gene resides on chromosome 17 and is complete in the CMT duplication (Matsunami *et al.*, 1992; Patel *et al.*, 1992; Timmerman *et al.*, 1992; Valentijn *et al.*, 1992a). In one family that displayed CMT the duplication incorporated only 460kb of chromosome 17. This region, while missing the majority of genes found in the more common duplication, does include the full PMP-22 sequence (Valentijn *et al.*, 1993) further implicating it as being significant.

To further corroborate PMP-22 as the target gene, a number of families have been identified in which the CMT phenotype is caused by point mutations in the gene - commonly in the putative transmembrane spanning regions. In one family the exact mutation seen in *trembler-J* has been identified (Valentijn *et al.*, 1992b; Roa *et al.*,

1993b,c). A 2 base pair frame shift in the first translated exon has been associated with HNPP (Nicholson *et al.*, 1994). And two point mutations have been identified with the more severe DSS phenotype (Roa *et al.*, 1993a).

The search for mutations in other myelin genes lead to the discovery of the site of mutations responsible for CMT-1B and CMT-X. The importance of genes such as P_O has been described. Giese *et al.* (1992) produced transgenic mice with a disrupted P_O gene. The resulting mice show deficiencies in motor co-ordination and tremors with occasional seizures. A severe but incomplete loss of myelin in the PNS is seen. The mutation site in CMT-1B has been localised to chromosome 1, the site of the human P_O gene. Missense mutations within the extracellular domain have been identified in the CMT-1B patients (Hayasaka *et al.*, 1993a,b). A 3 base pair in frame deletion has also been implicated (Kulkens *et al.*, 1993). Again mutations are occuring in functionally significant sites, since the extracellular domain of P_O is implicated in the homophilic interactions that maintain the myelin sheath.

The gene that carries the mutation for the X-linked form of CMT has been found to encode the gap junction protein Connexin 32, Cx32 (Bergoffen *et al.*, 1993). Cx32 is expressed in a number of different tissues (Dermietzel and Spray, 1993), however the effects of mutations within it are restricted to the PNS. In the PNS Cx32 expression is restricted to the Schmidt-Lanterman clefts and the paranodal loops of the myelin sheath (Bergoffen *et al.*, 1993a).

In addition, mutations in the gene for Krox-20 have demonstrated the involvement of this transcription factor in the disease process.

1.6.7 Transgenic Mouse Studies

The conformation of PMP-22 as the disease causing gene was achieved with the production of a number of transgenic animals. Overexpression of the gene and disruption of it gave rise to phenotypes corresponding to those seen in the human and mouse condition.

A rat model produced by Sereda *et al.* (1996) with three additional copies of the PMP-22 gene shows symptoms compatible with those of Charcot Marie Tooth disease. Heterozygous animals developed an abnormal gait and suffered tremor and transient seizures from 2 months of age. A loss of muscle strength was seen and the rats failed standard behavioural assays for motor performance. A decrease in the conduction properties of both sensory and motor nerves was observed. The myelin sheath in the peripheral nervous system was thin or absent and at 2.5 months onion bulbs began to form. The progressive nature of this model manifests itself in extensive onion bulb formation by 6 months of age. Motor nerves and large calibre axons were mainly affected. Myelin debris was occasionally observed.

An increase in the severity of phenotype was observed in a mouse model in which an additional 16 copies of the PMP-22 gene was incorporated (Magyar *et al.*, 1996). Whilst Schwann cells and axons had associated in the correct manner and a basement membrane was present, no compact myelin was formed. A two fold overexpression of PMP-22 mRNA was found, whilst levels of other myelin protein mRNAs were severely reduced. This increase in the amount of mRNA is in part due to an increase in the number of Schwann cells found. The abnormal proliferation of Schwann cells seen in the *trembler* and *trembler-J* mice was mirrored here. Behaviourally by 2 weeks of age mice show shivering, followed by an unsteady gait. By 2-3 months muscle atrophy occurs. Nerve conduction velocities are reduced. In the homozygous animal a complete loss of myelin was seen. Homozygotes never developed a normal gait and were paralysed by 4 weeks. Many died or had to be sacrificed before this time.

Overexpression of the human PMP-22 cDNA in transgenic mice has a less pronounced effect on Schwann cell development but the study demonstrates the apparent dose sensitivity of PMP-22 expression. Mice with two additional copies of the gene displayed a normal phenotype and little effect was seen. Animals with four copies of the human gene showed some hypomyelination at adulthood and an electrophysiological defect. However, no behavioural abnormalities were found. When seven or more copies of the human gene were incorporated a severe dysmyelinating phenotype was observed with an

abnormal gait and progressive paralysis. The differences between the severity of effects seen with the introduction of the human versus the murine gene may be due to inefficient functioning of the human gene in the mouse environment (Huxley *et al.*, 1996; 1998)

Mouse models of HNPP have also been produced. The disruption of the PMP-22 gene gives rise to a line of mice that show behavioral, electrophysiological and morphological abnormalities. Adlkofer *et al.* (1995) produced a mouse with such a null mutation. Heterozygous mice, with only one copy of the correct gene were phenotypically indistinguishable from wild type litter mates. Some individuals showed sporadic walking difficulties. They showed near normal nerve conduction velocities yet some myelin deficiencies occurred. The numbers of tomacula increased from 24 days to 10 weeks old and occasional onion bulbs were seen. No significant changes in axon number were observed. Homozygous mice begin to show walking difficulties with progressive paralysis of the hind limbs by post-natal day 14. Occasional tremor and convulsions were seen. Electrophysiological studies show a slowing of the NCV and increased motor latencies. At 24 days myelin thickenings, tomacula were observed, and the accompanying axons appeared compressed. Early stages of demyelination were seen, and large calibre axons were devoid of myelin. By 10 weeks of age tomacula were rare and onion bulbs became more prominent. Myelin degeneration was also seen.

1.7 Aims and Objectives

The peripheral myelin protein 22 has previously been implicated as an important component of the peripheral myelin sheath. It is localized to the Schwann cell and its expression follows that of other myelin proteins as it increases from birth concomitant with the extensive myelination of the peripheral nerves. Interaction between it, and the major myelin protein $P_{0,}$ is thought to maintain the compact structure of the mature myelin. When cells transfected with either protein were grown together, the two proteins were seen to co-localise at apposing membranes suggesting an interaction between them (D'Urso *et al*, 1999). In the disease state, disruption of either component that prevents the two proteins interacting or that alter the correct stoichiometry between them, result in abnormalities of myelin formation and behavioural phenotypes charactersied by tremors.

As highlighted previously, the regulation of PMP-22 is complex. Two alternative promoters P1 and P2 give rise to alternate transcripts identified by the presence of non-translated exons 1a or 1b respectively. The differential regulation of these promoters results in a defined pattern of expression. Activity of the P1 promoter identified by the presence of the exon 1a transcript is responsible for the expression of PMP-22 in Schwann cells of the peripheral nerve and is intimately involved in the myelination process. Exon 1b containing transcripts predominate at sites of non-neural expression of PMP-22.

The aim of this project was to produce a transgenic mouse that would express the reporter gene - green fluorescent protein (GFP) specifically in Schwann cells of the peripheral nervous system. The relevance of PMP-22 to disease, both in murine mutants *trembler* and *trembler-J* and the human peripheral neuropathies, combined with the myelin specific nature of the P1 promoter made it an interesting candidate to study the regulation of the expression of a reporter gene within the transgenic mouse system. This would allow the study of both PMP-22 expression and regulation during development and myelin formation and the myelination process itself.

The aim was to use the mouse model to address a number of issues. Despite the presence of a number of regulatory elements typical of a tissue specific promoter that is strongly regulated, the P1 promoter conferred only limited promoter activity in driving expression of a reporter gene *in vitro* (Suter *et al.* 1994). Within the *in vivo* environment it was hoped that the necessary signals would be present that would allow correct regulation of the P1 promoter. Description of the transgenes expression pattern during development will confirm the P1 promoter region as containing all the necessary elements for tissue and temporal specific regulation of PMP-22. *In vitro* study of Schwann cells derived from the transgenic mouse could be used to assess the influence of transcription factors or hormones on the regulation of the promoter.

The highly visual nature of the reporter gene will allow study into the dynamic behaviour of Schwann cells and neurons. Previous studies in which the description of the elaboration of the myelin sheath has been determined have relied on detailed electron microscopic examination. The absence of a requirement for co-factors and substrates of the GFP reporter gene will allow the visualisation of these processes in real time.

Chapter 2.

Materials and Methods

Chapter 2. Materials and Methods.

This chapter aims to give a detailed description of the materials and methods used throughout the following chapters. All chemicals used were of an analytical or molecular grade and were purchased from Sigma or BDH unless otherwise stated.

2.1 Solutions

Antibody Dilution Buffer

10% HIGS
1mg/ml bovine serum albumin
0.5% Triton-X-100
made up in PBS

Genomic DNA Extraction Lysis Buffer

50mM Tris-HCl pH8
100mM EDTA
0.5% SDS

2x HEPES Balanced Salt Solution

280mM NaCl
10mM KCl
1.5mM $Na_2HPO_4.2H_2O$
12mM dextrose
50mM HEPES, pH 7.05

Luria Bertani (LB) media

1% peptone
0.5% yeast extract
1% NaCl, pH 7

PBS

137mM NaCl
8.5mM $NaH_2PO_4.12H_2O$
1.5mM KH_2PO_4
2.7mM KCl

Plasmid DNA Extraction Solution 1

50mM Tris-HCl pH 7.5

10mM EDTA

100µg/ml RNase A

Plasmid DNA Extraction Solution 2

0.2M NaOH

1% SDS

Plasmid DNA Extraction Solution 3

1.32M potassium acetate pH 4

Plasmid DNA Extraction Solution 4

80mM potassium acetate

8.3mM Tris-HCl pH 7.5

40µM EDTA

55% ethanol

SOC Bacterial Growth Media

2% peptone

0.5% yeast extract

10mM NaCl

2.5mM KCl

10mM $MgCl_2$

10mM $MgSO_4$

20mM glucose, pH 7

Southern Blot Solutions:

Denaturing Solution

1.5M NaCl

0.5M NaOH

100% Denhardts Solution

2% BSA

2% Ficoll

2% polyvinylpyrollidene

Neutralising Solution

1.5M NaOH

0.5M Tris-HCl pH 7.2

0.001M EDTA

Pre-Hybridisation Solution

5x SSPE

5x Denhardts Solution

0.5% SDS 1

100μg/ml sonicated salmon sperm DNA

20x SSC buffer

3M NaCl

0.3M sodium citrate

100 x SSPE

3.6M NaCl

0.2M Na2PO4

0.02M EDTA

Superbroth

33g peptone

20g yeast extract

7.5g NaCl

1g glucose in 1l

1x TAE Buffer

0.04M Tris-acetate

0.001M EDTA

T.E

10mM Tris-HCl

1mM EDTA pH 8

2.2 Isolation of Plasmid DNA

2.2.1 Mini – Prep Method

This procedure is a modification of that of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) as cited in Sambrook *et al.* (1989). A single colony of transformed bacteria was grown overnight in 5ml of Luria Bertani (LB) media with appropriate antibiotic selection. 1.5ml of the overnight culture was taken and the cells harvested by centrifugation at 12,000g for 1 minute. The supernatant was discarded and the cells resuspended in 200µl of resuspension solution (Solution 1: 50mM Tris-HCl pH 7.5, 10mM EDTA, 100µg/ml RNase A). 200µl of lysis buffer (Solution 2: 0.2M NaOH, 1% SDS) was then added and the solutions mixed by inversion until the suspension became viscous indicating cell lysis. 200µl of neutralising solution (Solution 3: 1.32M potassium acetate, KOAc pH 4) was then added and the solutions mixed by gentle vortexing. The sample was centrifuged at 14,000g in a bench top microfuge for 1 minute to pellet bacterial debris. The supernatant was transferred to a clean 1.5ml microcentrifuge tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) added. Following centrifugation at 14,000g for 10 minutes the aqueous phase was removed and DNA precipitated by the addition of 2 volumes of 100% ethanol and 0.1 volume of 3M sodium acetate (NaOAc) as described below (section 2.3.1.1).

Alternatively 500µl of Promega Magic Mini-Prep DNA purification resin were added to the supernatant removed after the addition of solution 3. This was incubated at room temperature for 5 minutes with occasional inversion. The solution was then pipetted into a syringe barrel attached to a Magic Mini-Prep column. The solution was injected into the column then washed through with 4ml of column wash solution (Solution 4: 80mM potassium acetate, 8.3mM Tris-HCl pH 7.5, 40µM EDTA, 55% ethanol). The column was centrifuged for 1 minute at 12,000g and then air dried for 5 minutes. Following transfer to a clean 1.5ml microcentrifuge tube the DNA was eluted with 100µl sterile milli Q water pre-heated to 60°C. This was added to the column which was incubated for 5 minutes at room temperature prior to elution by centrifugation at 12,000g for 1 minute.

2.2.2 Midi-Prep Method

A fresh 5ml overnight culture of transformed bacteria in LB was subcultured at 1 in 200 into 200ml of LB and grown overnight at 37°C with antibiotic selection. Cells were pelleted by centrifugation at 6000g for 20 minutes (in a JA14 rotor suitable for use with Beckman J2-HC centrifuge) and resuspended in 10ml of solution 1. They were then lysed with 10ml of solution 2 and neutralised with the same volume of solution 3. The sample was centrifuged for 20 minutes at 16,000g and the supernatant removed to a clean tube where an equal volume of isopropanol was added and the solution mixed by inversion. DNA was pelleted by centrifugation at 14,000g for 20 minutes and allowed to air dry before resuspension in 2ml of T.E. (10mM Tris-HCl and 1mM EDTA pH 8), after which 10ml of Promega Wizard MidiPrep DNA purification resin was added. The slurry was run into a Wizard MidiPrep DNA purification column and washed with 30ml of column wash solution 4 followed by 5ml of 80% ethanol. The column was spun briefly to aid drying and allowed to air dry before DNA was eluted. 1ml of T.E. was added to the column, this was then incubated at room temperature for 5 minutes before being spun for 1 minute at 2500 rpm to recover the DNA solution. The elution step was repeated to ensure DNA recovery was maximised.

2.2.3 Cesium Chloride Purification of Plasmid DNA - Continuous Gradient

500ml bacterial cultures were grown up to an OD_{600} of 0.5–1.0 in superbroth (Howe, 1973; 33g peptone, 20g yeast extract, 7.5g NaCl, 1g glucose in 1l)

Bacteria were pelleted by centrifugation at 6000g for 20 minutes and the cells lysed using 20ml of solutions 1, 2 and 3 as described in section 2.2.2. Following isopropanol precipitation the DNA pellet was resuspended in 4ml of T.E.

For every 1ml of DNA solution 1g of CsCl was added and for every 10ml of this solution 0.8ml of ethidium bromide (10mg/ml) was added. The solution had a final density of 1.55g/ml with an ethidium bromide (EtBr) concentration of approximately 740μg/ml. The solution was centrifuged for 5 minutes at 8000g to separate bacterial proteins that form a scum layer at the surface. The DNA solution was removed and placed in Beckman Quick seal centrifuge tubes suitable for a Beckman Ti65 rotor. The density gradients were then centrifuged at 45,000 rpm in a Beckman VTi65 ultra centrifuge for 16 hours.

Two distinct bands of DNA were visualised using a transilluminator (UVP Inc.). The upper band consisted of bacterial chromosomal DNA and nicked circular plasmid DNA while the lower band contained the closed circular plasmid DNA. A 21 gauge hypodermic needle was inserted in to the top of the tube to allow air to enter. After the side of the tube had been wiped clean with a swab of ethanol an 18 gauge needle was inserted, bevelled side up, through the side of the tube beneath the layer of closed circular plasmid DNA which was then withdrawn.

2.2.3.1 Removal of Ethidium Bromide

EtBr was removed from DNA samples by isoamyl alcohol extraction. An equal volume of isoamyl alcohol was added, the sample vortexed and centrifuged at 1500 rpm for 3 minutes in a bench top centrifuge. The lower aqueous phase was transferred to a clean microcentrifuge tube and the step repeated 4 or 5 times until both phases were clear. The solution was subsequently dialysed over 24-48 hours against several changes of T.E. to remove the CsCl. The purified DNA was quantitated spectrophotometrically as described (section 2.4.1).

2.3 Genomic DNA Extraction Methods

2.3.1 Phenol Chloroform Extraction

2-4mm sections of mouse tail were incubated in 0.7ml of lysis buffer (50mM Tris-HCl pH8, 100mM EDTA, 0.5% SDS) with 10µl of 25mg/ml Proteinase K for at least 4 hours at 55°C until digested. An equal volume of phenol pH 7.5 was added and the sample shaken vigorously. Following a centrifugation step of 10 minutes at 12,000g the aqueous phase was removed to a clean microcentrifuge tube and 0.6ml phenol:chloroform:isoamyl alcohol (25:24:1) added. The sample was mixed and centrifuged for a further 5 minutes at 12,000g. The aqueous phase was again removed and the DNA isolated from it by ethanol precipitation as described below.

2.3.1.1 Ethanol Precipitation

DNA was precipitated by the addition of 2 volumes of 100% ethanol and 0.1 volume of 3M sodium acetate (NaOAc) pH 4.8. The sample was mixed and incubated at –20°C for at least 30 minutes then centrifuged at 12,000g for 10 minutes. The DNA pellet was washed with 70% ethanol with a 5 minute centrifugation at 12,000g. The

supernatant was discarded and the pellet air dried and subsequently resuspended in T.E. Following genomic extraction, DNA was resuspended at 4°C overnight or at 65°C for one hour.

2.3.2 Promega Wizard Genomic DNA Purification

Genomic DNA was extracted using the Promega Wizard Genomic DNA Purification system following the manufacturers instructions. Briefly a 2-4mm tail sample was incubated at 55°C for at least 3 hours in 600µl of EDTA/nuclei lysis solution (500µl of nuclei lysis solution was added to 120µl 0.5M EDTA chilled on ice before use) with 17.5µl of 20mg/ml Proteinase K solution. Following this 200µl of protein precipitation solution was added to the sample and the solution vortexed for 20 seconds. The sample was incubated on ice for 5 minutes before the precipitated protein was pelleted by centrifugation at 13-16,000g for 4 minutes at room temperature. The supernatant was transferred to a clean tube, and 600µl of isopropanol added. The tube was gently inverted until threads of DNA could be seen. The DNA was pelleted at 13-16,000g for 1 minute in a bench top centrifuge. The supernatant was discarded and the DNA washed in 600µl of 70% ethanol. Following repeated centrifugation the DNA pellet was air dried and subsequently rehydrated in 50µl of T.E. at 4°C overnight, or 65°C for one hour.

2.3.2.1 Modification of Wizard Genomic Purification for the Extraction of Genomic DNA from Cultured Cells

Adherent cells were trypsinised from the surface of the culture dish. The cells were then collected by centrifugation at 13,000g for 10 seconds. The supernatant was removed leaving the cells in 10-50µl of residual media. The pellet was resuspended in 200µl of PBS. Following the addition of 600µl of nuclei lysis solution the mixture was pipetted to lyse the cells. Protein precipitation solution was then added and the procedure progressed as described in section 2.3.2.

2.4 Quantification of DNA Solutions

2.4.1 Spectrophotometric Determination

The absorbencies of diluted aliquots of DNA samples were determined at 260nm on a Beckman DU7500 spectrophotometer. An absorbance of 1.0 at 260nm is equivalent to a concentration of 50μg/ml of double stranded DNA and from this DNA concentrations could be calculated.

2.4.2 Qualitative Analysis of DNA Solutions

The optical density of each sample was also measured at 280nm. A ratio of 1.8 – 2.0 between OD_{260} and OD_{280} was indicative of an uncontaminated DNA sample.

2.4.3 Comparative Determination of DNA Concentration

Comparison of the DNA sample with that of known concentrations of digested λ DNA on an agarose gel gave an estimate of the DNA concentration.

2.5 DNA Manipulations

2.5.1 RNase A Treatment

RNA was removed from DNA solutions by the addition of RNase A to a final concentration of 20μg/ml. Denatured RNA was subsequently removed by ethanol precipitation (section 2.3.1.1).

2.5.2 Restriction Endonuclease Digestion of DNA

Plasmid DNA was digested at concentrations of 10–100ng/μl in a total volume of 10-50μl with 5 units of restriction endonuclease, 0.1 volume of the appropriate 10x enzyme buffer made up to the final volume with sterile water. The reaction was incubated at the recommended temperature, for the majority of restriction enzymes this is 37°C, for one hour.

For Southern Blot analysis of genomic DNA a total of 10μg of DNA was digested in a 50-100μl volume with 40 units of restriction enzyme for 4-5 hours at the recommended temperature. For further analysis by gel electrophoresis the digest was subsequently concentrated by ethanol precipitation as previously described (section 2.3.1.1) and resuspended in a volume of 30μl T.E.

2.5.3 Electrophoretic Separation of DNA Fragments

Gel electrophoresis was performed using 1-2% agarose gels (1x TAE buffered, 0.04M Tris-acetate, 0.001M EDTA). The concentration of agarose was varied according to the size of fragment to be analysed (Sambrook *et al.*, 1989). Generally, 0.8% agarose gels were used. These efficiently separate linear DNA between 0.8–10.0 kb. PCR fragments were isolated on 1.2% gels giving a resolution of 0.4 –6.0 kb size fragments.

DNA fragments were visualised using a trans-illuminator (UVP Inc.) or Gel Doc 1000 imaging system (Biorad).

2.5.3.1 DNA Fragment Isolation: Qiaex II Agarose Gel Extraction

DNA to be recovered following electrophoresis was removed in the smallest possible volume of agarose and isolated by the following protocol.

The Qiagen Gel Extraction kit was used according to the manufacturer's guidelines. Briefly, 3 volumes of buffer QX 1 was added to 1 volume of agarose. This was incubated at 50°C for 10 minutes with 10µl of Qiaex II particles. The sample was centrifuged for 30 seconds and the supernatant discarded. The pellet was resuspended in 500µl of buffer QX1 and spun for 30 seconds at 14,000g in a bench top microfuge. The pellet was then washed twice in the supplied buffer PE and allowed to air dry for 10-15 minutes. The pellet was resuspended in 20µl of T.E., spun for 30 seconds and the DNA solution removed to a clean microcentrifuge tube. The elution step was repeated to ensure maximum recovery of DNA.

2.5.4 End Filling of DNA Fragments

The 3' recessed ends of a restriction fragment were converted to blunt ends by the following method which utilises the 3' - 5' exonuclease activity of the Klenow fragment of DNA polymerase I (Sambrook *et al.*, 1989). The DNA fragment (maximum of 500ng) was incubated in a final volume of 50µl with 5µl of 10x Klenow buffer (130mM KPO_4, 6.5mM $MgCl_2$, 1mM dTT, 0.032mg/ml BSA), 1.4µl of stock dNTP's (each at 1.2mM) and 2 units of Klenow enzyme per 1µg of DNA. This was incubated at room temperature for 30 minutes, EDTA was added to a final concentration of 10mM, and incubated for 10 minutes at 75°C to inactivate the enzyme. Buffer and enzyme contaminants were then removed using a simple

modification of the method given in section 2.5.3.1. from which the incubation at 50°C for 10 minutes was omitted.

2.5.5 Dephosphorylation of Plasmid Vector DNA

Dephosphorylation of vector DNA prevents its re-circularisation during ligation. Up to 20µg of vector DNA was incubated with 5µl of 10x dephosphorylation buffer and 1µl of calf intestinal alkaline phosphatase (CIP) in a final volume of 50µl at 37°C for 30 minutes. The enzyme was then inactivated by the addition of EDTA to 5mM and incubation at 75°C for 10 minutes. Buffer and enzyme contaminants were then removed using the modification of the Qiagen Gel Extraction kit described in section 2.5.4.

2.5.6 Ligation of DNA

Bacteriophage T4 DNA ligase was used to ligate together DNA fragments and linearised plasmid vector with either compatible cohesive or blunt termini. The fragments of insert and vector DNA were mixed at a molar ratio of 3:1 respectively with a total combined amount of 100ng of DNA. A 0.1 volume of 10x ligation buffer (0.66M Tris-HCl pH 7.5, 50mM $MgCl_2$, 50mM DTT, 10mM ATP [stock ATP solution 100mM disodium salt, pH 7.0 adjusted by addition of 0.1 volume of Tris-HCl pH 9.5 and stored at –20°C]) was added with 1 unit of T4 DNA ligase for cohesive termini. For blunt ended termini 3 units of ligase were added. The reaction was then incubated at 4°C overnight. The ligation mixture was then used to transform competent *E. coli* cells as described in section 2.5.8.

Control reactions were carried out in tandem to assess the efficiency of each stage of the protocol. Ligation reactions that omitted the addition of T4 DNA ligase were performed to ensure that in the initial stages the DNA components were fully digested. Any transformant colonies resulting from this reaction would indicate that some circular DNA remained. Reactions were carried out in which vector DNA only was added to the reaction. In one instance the non-phosphorylated vector DNA was used. In another only the CIP treated, dephosphorylated vector was present in the reaction. Comparison of the number of transformed colonies in each will give an indication of the efficiency of the dephosphorylation reaction. Finally a control to the transformation step was performed in which known circular plasmid DNA was transformed. This ensured that the competent cell aliquot used was viable.

2.5.7 Competent Cells

2.5.7.1 Calcium Chloride Competent Cells

The method employed was a variation of that used by Cohen *et al.* (1972) as cited in Sambrook *et al.* (1989). Briefly a 5ml aliquot of LB media was inoculated with a single colony of *E.coli* and incubated overnight at 37°C with appropriate antibiotic selection. This was subcultured at a 1 in 100 dilution in 1l of LB broth and grown until the optical density at 600nm (OD_{600}) reached between 0.5 and 1.0. The cells were chilled on ice for 10 minutes and pelleted at 4000g for 5 minutes at 4°C. The pellet was then resuspended in 50ml of ice cold sterile $CaCl_2$ (100mM, pH 8) and stored on ice for 15 minutes before the centrifugation step was repeated. The cell pellet was resuspended in 6.66ml of ice cold $CaCl_2$ (100mM, pH 8) and dispensed into 0.1ml aliquots. These were kept on ice until immediate use (within 24 hours) or alternatively snap frozen in 20% glycerol in liquid nitrogen and stored at -80°C for subsequent use.

2.5.7.2 Electrocompetent Cells

One litre of LB was inoculated with a single colony of fresh *E. coli* and incubated at 37°C until the OD_{600} was 0.5 – 1.0. The culture was chilled on ice for 15-30 minutes then the cells pelleted at 6000g for 15 minutes at 4°C. The pellet was resuspended in one litre of ice cold sterile distilled water. The chilling and centrifugation step was repeated and the cell pellet resuspended in 0.5l of cold sterile water. Following a further cycle of incubation on ice and centrifugation the subsequent cell pellet was resuspended in 20ml of 10% glycerol. A final centrifugation was performed and the cells were resuspended in 2-3ml of 10% glycerol. 50µl aliquots were frozen on dry ice and stored at –80°C for up to 6 months.

2.5.8 Transformation of Competent Cells with Circular Plasmid DNA

2.5.8.1 Calcium Chloride Transformation

An aliquot of $CaCl_2$ competent cells was resuspended in 900µl of ice cold sterile 0.1M $CaCl_2$. A 100µl aliquot was removed from this and up to 100ng of the plasmid DNA added. The aliquot was incubated on ice for 30-90 minutes before being heat shocked at 43.5°C for 45 seconds. The cells were then placed on ice for 2 minutes before the addition of 900µl of SOC (2% peptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose, pH 7), and then

incubated at 37°C for at least 30 minutes to allow cell recovery. 100µl of the cell suspension were spread across the surface of solid LB plates (LB with 1.5% agar with antibiotic selection appropriate to the cell strain and plasmid DNA) and the inverted plates were then incubated overnight at 37°C. The remainder of the transformed cell suspension was centrifuged briefly in a bench top centrifuge to pellet the cells. The majority of the supernatant was removed leaving approximately 100µl, the remaining cells were subsequently resuspended in this volume and plated on solid LB with antibiotic selection and incubated at 37°C overnight.

2.5.8.2 Electro-Transformation

1ng of DNA was added to 40µl of electrocompetent cells which were subsequently incubated on ice for 60-90 seconds, transferred to a chilled cuvette (2mm gap width) and electroporated (Bio-Rad Gene Pulser electroporator set to 2.5kVolts, 100 Ohms with a capacitance value of 2.5). An indication of transformation efficiency was established by noting a time constant of approximately 4.7. Following electroporation 1ml of SOC was added and the cells were allowed to recover at 37°C for an hour. Aliquots were then plated on LB as described above (section 2.5.8.1) and grown at 37°C.

2.5.9 Blue/ White Selection Screening.

This technique is used to identify recombinant, successfully ligated clones within appropriate vector backbones. A series of plasmid vectors have been developed that contain the sequence for the first 146 amino acids of the β-galactosidase gene. When transformed into appropriate host cells containing the carboxy terminal of the β-galactosidase gene the components can associate via α-complementation to form an enzymatically active protein (Ullman *et al.*, 1967). In the presence of the substrates β-D-isopropyl-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) such Lac Z+ bacteria exhibit a blue colour (Horwitz *et al.*, 1964). The insertion of a foreign DNA fragment into the *lacZ* gene of the plasmid gives rise to an amino terminal incapable of α complementation and will result in white bacterial colonies.

The ligation reaction was transformed into appropriate bacterial host strains such as JM109. Previously prepared solid LB plates with the correct antibiotic selection were spread with 50µl of 50mg/ml X-Gal and 100µl of 0.1M IPTG. These components

were allowed to absorb into the plate for at least 30 minutes before the transformed cells were plated out.

2.6 DNA Sequencing

Plasmid DNA for DNA sequencing was prepared using the Promega Wizard miniprep kit as described previously (section 2.2.1). Sequencing of double stranded plasmid templates was performed by the dideoxy-sequencing method of Sanger *et al.* (1977) using the fluorescent dye linked universal primers SP6 and T7. Custom primers were used in conjunction with the Applied Biosystems Taq dideoxy terminator cycle sequencing kit. Sequencing was performed in both directions using either an Applied Biosystems 373 DNA Sequencer or an ABI Prism 377 XL DNA Sequencer.

2.7 Transfection Protocols

The ability of plasmid constructs to express the desired gene can often be assessed by introducing the plasmid into an appropriate cell type. This can be achieved by any of the appropriate methods.

2.7.1 Calcium Phosphate Transfection

24 hours before transfection cells were harvested and plated at a density of $1x10^5$ – $2x10^5$ cells/cm^2 in 35mm culture dishes and incubated at 37°C in a 5% CO_2 atmosphere. For each dish 110µl of DNA (40µg/ml in T.E.) were added to 125µl of 2x HEPES buffered saline or HBS (280mM NaCl, 10mM KCl, 1.5mM $Na_2HPO_4.2H_2O$, 12mM dextrose, 50mM HEPES, pH 7.05, filter sterilised through a 0.22 micron filter). Subsequently15.5µl of 2M $CaCl_2$ were added dropwise with gentle mixing. Following incubation at room temperature for 20-30 minutes the DNA precipitate formed was resuspended by trituration and added to the media above the cells. After incubation of the cells with the DNA complex for at least six hours (optimised for each cell type), the cells were washed with PBS and fresh media added. The cells were assayed for expression of the transfected construct 48-72 hours later.

2.7.2 Lipophilic Transfection Reagents – Fugene

24 hours before transfection the cells were plated at 1-3x10^5 cells/35mm dish. For each dish 97µl of serum free culture medium was placed in a tube, and 3µl of Fugene (Boehringer-Mannheim) were then added directly and the solution left to stand for 5 minutes. Care was taken to avoid contact with the walls of the tube and so avoid absorption of the reagent to the plastic. The complex was then added dropwise to a second tube containing 1-2µg of the DNA to be transfected and gently mixed. Following a 15 minute incubation at room temperature the mixture was slowly introduced to the media above the cultured cells. Unless necessary for subsequent experiments the reagent was not removed from the cells prior to assay for the expression of the construct.

2.7.3 Lipophilic reagent - Lipofectamine

Cells were subcultured 24 hours before transfection as described previously (2.7.2). For each 35mm culture dish 1-2µg of DNA were suspended in a final volume of 100µl with serum free media. In a second tube 10µl of the transfection reagent Lipofectamine (Life Technologies) was added to 90µl of serum free media. The two solutions were combined and incubated together for 15-45 minutes before 0.8ml of serum free media were added. Media was removed from the cells and replaced with 1ml of the DNA containing solution. Cultures were incubated with the DNA complex for 6 hours at 37°C, 5% CO_2 before it was replaced with 2ml of growth media. The cells were assayed for expression after 48-72 hours.

2.7.4 Transfection via Electroporation

Cells were trypsinised from the culture dish and suspended at a density of 2-5x10^6 cells/ml. 10µg of DNA in a volume of less than 40µl was added to 400µl of the cell suspension. This was placed in a 4mm cuvette and pulsed at 220-300V (Genetronics BTX, Electro Cell Manipulator), optimised for each cell type. 1ml of growth media was immediately added and the cells re-plated in a 35mm culture dish.

2.8 Southern Blotting Techniques

2.8.1 Denatured Sonicated DNA

Salmon sperm DNA was suspended in water at a concentration of 10mg/ml and sheared by repeatedly being passed through a 17 gauge hypodermic needle. The DNA was then denatured in a boiling water bath for 10 minutes followed by quenching on ice for 5 minutes before use or storage at -20°C.

2.8.2 Preparation of Radiolabelled Probes.

DNA fragments were labelled with [^{32}P] dCTP 10μC/μl (Amersham) using the random primer method of the Amersham "Rediprime" labelling kit. 25ng of probe DNA was diluted to a final volume of 45μl with T.E. and denatured in a boiling water bath for at least 5 minutes. The volume was adjusted if necessary to 45μl and was added to a Rediprime kit aliquot. 5μl of [^{32}P]-dCTP (10μC/μl) was added and the reaction incubated at 37°C for 20-30 minutes. The labelled DNA was then denatured once more in a boiling water bath for 5 minutes and quenched on ice for a further 5 minutes. This was then added directly to the pre-hybridisation solution (section 2.8.4).

2.8.3 Transfer of DNA to Nitrocellulose Membranes

The following method is a modification of that of Southern (1975). DNA was transferred from an agarose gel to a Hybond-C nitrocellulose membrane (Amersham) following the procedures below. The agarose gel containing the DNA samples was photographed with a fluorescent calibration rule. The genomic DNA within the agarose gel was depurinated in 0.25M HCL for 15 minutes then rinsed twice with distilled water. The gel was soaked in an excess volume of denaturing solution (1.5M NaCl, 0.5M NaOH) for 45 minutes followed by two distilled water rinses and then soaked in neutralising solution (1.5M NaOH, 0.5M Tris-HCl pH 7.2, 0.001M EDTA) for 45 minutes with gentle shaking. After further distilled water washes the gel was then blotted overnight.

A platform was placed over a reservoir of 20x SSC buffer (3M NaCl, 0.3M sodium citrate). A wick of 3MM Whatman filter paper was placed over this, its ends extending into the reservoir, and soaked with buffer. The agarose gel was placed, well-side down on top of the filter paper, and any air bubbles were gently removed.

A piece of pre-wetted nitrocellulose membrane, cut to the correct size was then placed onto the gel avoiding air bubbles. Once in place the membrane was not moved. Two additional pieces of buffer soaked Whatman 3MM filter paper and a stack of absorbable hand towels all cut to the same size as the gel were then placed over the membrane. The ensemble was then covered with a 0.5kg weight and left for at least 16 hours to allow DNA transfer after which time the apparatus was dismantled. The membrane and gel were removed together, placed membrane down on a piece of clean filter paper. The position of the wells was marked on the membrane before the gel was discarded. The membrane was washed in 2x SSC before air drying. The DNA was fixed to the membrane by baking at 80°C for 2 hours.

2.8.4 Hybridisation of Radiolabelled Probes to Southern Blots.

Hybridisation was carried out in a Techne Hybridisation tube in a Techne Hybridiser HB-1 oven. The membrane was incubated in 25ml of pre-hybridisation solution (5x SSPE [100x SSPE: 3.6M NaCl, 0.2M Na2PO4, 0.02M EDTA] 5x Denhardts solution [100% Denhardts: 2% BSA, 2% Ficoll, 2% polyvinylpyrollidene], 0.5% SDS and 100µg/ml sonicated salmon sperm DNA pre-heated to 65°C), for 6 hours at 65°C. A denatured ^{32}P-dCTP radiolabelled probe was then added and the filter hybridised overnight.

The probe solution was removed and the filter washed twice with pre-heated 2xSSPE, 0.5% SDS for 10 minutes at 65°C. This was followed by one wash with 1xSSPE, 0.5% SDS and a higher stringency wash with 0.1xSSPE, 0.5% SDS both at 65°C. The level of non-specific hybridisation was assessed using a Geiger counter following each wash. Finally the filters were wrapped in clingfilm and hybridisation bands detected as described below.

2.8.5 Detection of Hybridised Bands

The filter was enclosed in clingfilm and exposed to Fuji RX-100 X-Ray film within an Amersham Hypercassette at -80°C for at least 48 hours. Following this the position of the filter was marked on the photographic film which was subsequently developed in Ilford Phenisol High Contrast film developer and fixed with Kodak Unifix diluted according to manufacturer's instructions. If the signal detected was weak the filter was exposed to film for up to five days. The distance of the molecular

weight markers from the well was determined from the image of the original gel calibrated with a fluorescent ruler, and compared to the position of the radioactive band on the film to resolve the size of any hybridising fragments.

2.9 General Tissue Culture Techniques

2.9.1 Aseptic Techniques

All tissue culture work was carried out in a designated tissue culture unit. Solutions were prepared with autoclaved water and filter sterilised before use. All experiments were performed in a class II Microflow Biological Safety Cabinet and items sterilised with 70% ethanol before entry into the hood.

2.9.2 Subculturing Cells

Confluent flasks or 35mm culture dishes (Greiner) were subcultured as follows. The growth media was removed and replaced with sterile PBS to wash the remaining media from the cells. This was removed and replaced with 1ml or 0.3ml respectively of 0.25% trypsin. The cells were returned to a 37°C incubator, 5% CO_2 (LEEC) and observed until they detached from the base of the culture vessel. An excess volume of serum containing media was then added and the cells harvested from the media by centrifugation at 1000 rpm for 5 minutes. The cell pellet was washed with growth media. A further 1ml of fresh media was then added and the cells dissociated into single units by trituration through a fine pipette tip or hypodermic syringe. The cell density was then calculated as described below before aliquots were plated.

2.9.3 Cell Counting

To determine the density of viable cells prior to plating, equal volumes of 0.6% trypan blue and the cell suspension were mixed. An aliquot was removed and injected into the chamber of a gridded haemocytometer. Under a stereoscopic microscope the number of viable cells, those that have not taken up the blue dye, in the five subdivided sectors were counted. The average was taken from this and multiplied by 2 to account for the dilution factor to determine the number of cells x 10^4/ml. The density at which cells were then plated was dependent upon the cell type and subsequent procedures.

2.9.4 Treating Coverslips for use in Tissue Culture

Coverslips were immersed in excess xylene for 24 hours with gentle agitation. This was replaced with acetone for 30 minutes and then 100% ethanol for 24 hours. Coverslips were washed several times in distilled water until they washed clear and then further soaked in distilled water for 1-2 hours. They were then immersed in 100% ethanol for at least 1-2 hours before use, and were subsequently maintained in 100% ethanol.

2.9.4.1 Brief Treatment of Coverslips

Coverslips were treated with xylene as above overnight. This was replaced with acetone for 30 minutes and then 100% ethanol for 60 minutes before washing with distilled water. Coverslips were soaked first in distilled water for 1 hour then 100% ethanol for at least 1 hours before use. Coverslips were stored as above (2.9.4).

2.9.5 Collagen Coated Coverslips

Treated coverslips were coated with collagen and poly-d-lysine before use. Collagen Type I from rat tail (Sigma) was dissolved at 0.5mg/ml in 0.1% acetic acid and stored in 1ml aliquots at –80°C. Equal volumes of poly-d-lysine (Sigma, 100µg/ml filter sterilised) and the prepared collagen were mixed and 100µl spread across the surface of each coverslip. This was allowed to air dry before being sterilised with UV light for 30 minutes.

2.9.6 Alternative Substrates for Coating Coverslips

Clean coverslips were coated with 100µl of collagen (0.25mg/ml), poly-lysine (50µg/ml) and laminin, components of the endogenous basal lamina and allowed to air dry. The commercially available, growth factor reduced basement membrane, Matrigel (Becton Dickenson) was diluted to a concentration of 0.148mg/ml with a consistency suitable to lay a fine film across the surface of the coverslip. Excess was applied to the coverslip, after an hour this was removed and the coverslip was allowed to air dry.

2.10 Conditions for the Maintenance of Cells *In Vitro*

A number of common cell lines have been used to express different plasmid constructs through transfection protocols. These are described below, with the conditions needed for their successful culture.

2.10.1 SCL4.1/F7

This transformed Schwann cell line was produced by pulsing primary rat Schwann cells with the mitogen, cholera toxin (Haynes *et al.*, 1994).

The cells are maintained in DMEM with low glucose and glutamax (Gibco) supplemented with 10% foetal bovine serum (FBS, Sigma). See Figure 2.10 C

2.10.2 Primary Myoblasts

Primary rat myoblasts were provided by colleagues. They were prepared following the trypsinisation and collagenase digestion of the hind limb muscle of neonatal rat. Cells were cultured on 1% gelatin coated plates to 90-95% confluency in DMEM containing 10% FBS and 2mM glutamine.

In preparation for transfection of the myoblasts the cells were pre-plated to remove any residual fibroblasts from the culture. Confluent flasks of the cells were trypsinised one day prior to the scheduled transfection and pre-plated in a 60mm petri dish with no gelatin coat for 25-30 minutes. Presumed fibroblasts adhered to the substrate leaving a suspension of myoblasts that were then collected and plated on gelatin coated dishes. See Figure 2.10 D

2.10.3 Fibroblasts

Primary human skin fibroblast cultures were donated by colleagues within the laboratory. Their growth media comprised DMEM with glutamax and 10% FBS. Figure 2.10 E

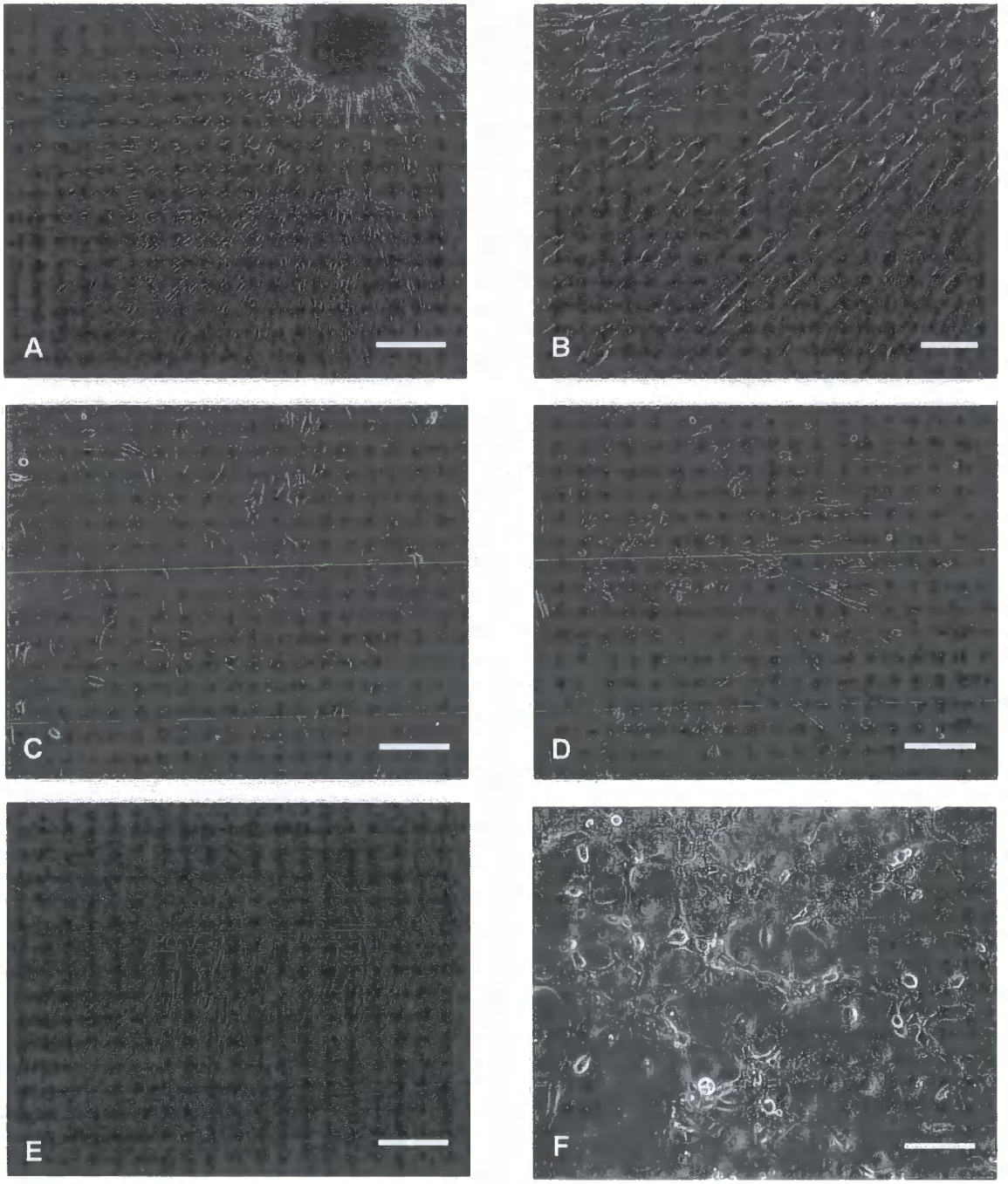
2.10.4 COS-7

COS-7 cells are derived from the CV-1, African Green monkey kidney cell line. They have been transformed by an origin-defective mutant of SV40 (Gluzman, 1981). These cells are widely commercially available.

They are maintained in Dulbecco's modified eagle's medium (DMEM) with glutamax (Gibco) and 10% foetal bovine serum (FBS, Sigma). See Figure 2.10 F.

Figure 2.10

Phase contrast images of cell types used throughout. **A** and **B.** Primary co-culture from the dorsal root ganglia with neuronal and Schwann cell outgrowth. Scale bar 200μm and 50μm respectively. **C.** SCL4.1/F7 transformed Schwann cell line. **D.** Preparation from neonatal rat muscle showing the presence of myotubes. **E.** Primary skin fibroblasts. **F.** COS-7 cell line derived from the kidney of the Green monkey. Scale bar 200μm


A
B
C
D
E
F

2.11 Preparation of Primary Cells

The use of animals in all procedures was carried out in accordance with Home Office guidelines under the project licence 60/2093 and personal licence 60/6195.

2.11.1 Schwann Cells Isolated from the Sciatic Nerve of Neonatal Animals.

This method is based on that of Brockes *et al.*, 1979.

2.11.1.1 Preparation of the Cells.

Newborn mice of between 4 and 5 days old were sacrificed by cervical dislocation. The lower abdomen was serilised with 70% ethanol and the skin over the leg retracted to reveal the sciatic nerve beneath the muscle. The sciatic nerve was removed from each pup from the lumbar spine to the knee joint and transferred to Hanks balanced salt solution (HBSS, Sigma).

When all the nerves had been collected they were removed from the HBSS and as much as possible of the residual media was removed. Spring scissors were used to mince the nerves to 0.5mm lengths and the tissue was then placed in a sterile 1ml tube.

The tissue was incubated with 0.5ml of collagenase B (1mg/ml, Boehringer-Mannheim) at 37°C for 5 minutes. The collagenase was removed and replaced with 1ml of 0.25% trypsin and further incubated at 37°C for 15-20 minutes.

The enzyme activity was arrested by the addition of 0.1ml heat inactivated FBS. The tissue was further dissociated by trituration with a 21 or 23 gauge hypodermic syringe. The contents were allowed to settle for one minute then the supernatant was removed to a sterile universal tube with 2ml of growth media (88ml DMEM, 10ml FBS, 2ml 0.25M glutamine, per 100ml). The suspension was centrifuged at 1,600 rpm for 3 minutes. The cell pellet was resuspended in growth media and split between 3 coated 35mm dishes. These were incubated at 37°C in a 5% CO_2 atmosphere. Cell counting is not necessary at this point as the culture contains a mixed population of cells.

2.11.1.2 Post Plating Maintenance of the Cultures

After one day in culture 1μm cytosine arabinoside was added to prevent the growth of fibroblasts.

After 3 days in culture the media was replaced with fresh growth media. At 5 days post plating a further 1μm of cytosine arabinoside was added. This was removed two days later, and the cells subsequently maintained in growth media.

2.11.2 Dorsal Root Ganglion Preparation of Schwann cells and Neurons in co-culture.

This protocol is based on that of Wood *et al*, 1976.

A 15 day pregnant mouse (day of plugging day 0) was sacrificed by cervical dislocation. The abdomen was sterilised with excess 70% ethanol and an incision made through the skin and body wall to reveal the uterus. Following an incision through the cervix and detachment from the ovaries the uterus was removed to a petri dish containing HBSS.

The uterine walls were separated and the embryos released. They were immediately decapitated to ensure humane death.

The internal viscera of each embryo was removed allowing ease of access to the spinal column. A pair of fine forceps was used to split the vertebrae along the length of the spine. From here the spinal cord could be lifted out with the ganglia intact. Under a dissecting microscope the ganglia were picked from the spinal cord and plated in coated 35mm culture dishes with 2ml of plating media (90ml basal media, 10 ml FBS, 50μl nerve growth factor [Sigma], per 100ml. Basal media, 500ml MEM [Gibco], 1.19g HEPES, 2.5g d-glucose adjust to pH 7.4, add 1.1g $NaHCO_3$ then filter sterilise). Any remaining ganglia, which failed to separate with the spinal cord, were removed from between the vertebrae with fine forceps and plated out. In general 8-12 ganglia were plated in each 35mm culture dish and three dishes could be prepared from 1 animal.

24 hours following plating the media was removed and replaced with 2ml of defined media (98ml of basal media, 1ml 200mM glutamine, 1ml N2 supplement, Gibco per 100ml.).

2.11.2.1 Dorsal Root Ganglion Neuron Cultures.

Cultures of pure neurons can also be prepared from dorsal root ganglia cultures. Ganglia were removed from embryos at 15 days of pregnancy, E15 as described above. One day after plating 10μM of the antimitotic reagents uridine and fluorodeoxyuridine were added to the media above the cells. The following day the media was replaced with defined media for 2 days. If Schwann cells were still present the anti-mitotic step was repeated. Bare neurons were maintained in defined media with nerve growth factor.

2.11.2.2 Removal of the dorsal root ganglia.

On occasion the neuronal cell bodies were removed from the co-cultures to give rise to cultures predominated by Schwann cells. The ganglia were removed by excision with a pulled Pasteur pipette tip. The debris was removed and fresh media was added. The cultures were monitored to ensure that all neuronal material had been removed.

Control experiments showed that within 2 days of removal the axons were found to have degenerated. Incubation of the cultures with NF68 monoclonal antibody to neurofilament showed the loss of all neuronal processes. (section 2.12).

The ganglia removed in this manner were capable of seeding new co-cultures following re-plating.

2.11.3 Crude Dissection of Different of Organs

Basic dissection of the heart, lung, liver and skeletal muscle from the hind limb of a transgenic embryo were carried out. The resulting pieces of tissue were dissociated by trituration through a fine bore syringe needle. The cell suspension from each was plated in serum containing plating media on collagen/poly-lysine coated cover slips (section 2.9.5).

2.11.4 Dissection of the Sciatic Nerve from Neonatal Mice

Neonatal mice were humanely sacrificed by cervical dislocation and the sciatic nerves removed. Briefly the skin was pulled back to expose the muscles of the hind limb. Fine forceps were used to gently part the individual muscles and expose the sciatic nerve. The nerve was separated from surrounding connective tissue and then removed from the lumbar spine to the knee joint. In older, larger animals a piece of

sterile cotton was pulled underneath the exposed nerve at one end and was tied around the nerve. Once the opposing end of the nerve had been severed from its attachment this prevented the nerve from recessing into the muscle. In younger, smaller animals this proved technically difficult and it was easier to maintain a hold on the fibre during the first cut. Once the opposite end was detached the nerve could then be removed and placed in PBS. One nerve from each mouse was gently teased apart using fine forceps until individual fibres were present. These were mounted in PBS and overlayed with a coverslip. This was then sealed with nail varnish. In this state the nerves were viable for analysis by confocal microscopy for approximately 48 hours. The remaining nerve was mounted in Tissue-Tek O.C.T Compound (Sakura) and frozen in liquid nitrogen before storage at –80°C.

2.12 Immunostaining Procedures

2.12.1 General Immunostaining Method

Primary cultures were washed twice with 2ml of phosphate buffered saline (PBS) at room temperature for 5 minutes before fixation in 2ml of 95% ethanol/5% acetic acid for 10 minutes at -20°C. Two subsequent PBS washes were followed by the addition of 100µl 10% heat inactivated goat serum (100% HIGS [ICN Biomedicals]: Goat serum was heat inactivated at a solution temperature of 65°C for 30 minutes. Dilutions to a final concentration of 10% were performed with PBS and stored in 1ml aliquots at –20°C) at room temperature for 15 minutes. 200µl of primary antibody (1:100 dilution with antibody dilution buffer [10% HIGS, 1mg/ml bovine serum albumin (BSA), 0.5% Triton-X-100] made up in PBS) were added to the coverslip with an incubation of 60 minutes at room temperature or overnight at 4°C. This was removed with four PBS washes before the addition of 200µl of fluorescent labelled secondary antibody (diluted 1:100 with antibody dilution buffer). The cells were incubated for 30 minutes in the dark at room temperature and then washed as before with four PBS washes. Coverslips were mounted on microscope slides with Mowiol (Calbiochem).

Commonly used secondary antibodies:

Tetramethyl-rhodamine-isothiocyanate (TRITC), Kirkegaard and Perry Inc.

Alexa-fluor 546, Molecular Probes.

2.12.2 Paraformaldehyde Fixation and Immunostaining

Primary cultures were washed twice with PBS and fixed with 4% paraformaldehyde, made up in PBS, for 20 minutes at room temperature. Post fixing was carried out with 100% methanol for 5 minutes at –20°C or sodium metaperiodate and L-glycine in PBS for 20 minutes at room temperature. Cells underwent two PBS washes before the addition of 100µl of 10% HIGS. Immunostaining was continued as described above (section 2.12.1).

Chapter 3.

Producing the Transgene Construct

Chapter 3: Producing the Transgene Construct

3.1 Introduction

3.1.1 Selecting the Appropriate Promoter

Peripheral myelin protein 22 (PMP-22) has been demonstrated to provide a crucial role in the formation and maintenance of the peripheral myelin sheath. Its temporal expression pattern is equivalent to that of other myelin proteins rising from birth and modulated by loss of axonal contact during nerve injury. Following nerve crush injury it has been well documented that proteins involved in the maintenance of the myelin sheath are downregulated. PMP-22 was originally identified using a strategy based upon the differential expression of transcripts following nerve crush injury. The cDNA sequence for two molecules CD25 and SR13 were identified (Spreyer *et al.*, 1991; Welcher *et al.*, 1991). The two transcripts were shown to encode for the same 22 kD glycoprotein (Snipes *et al.*, 1992) subsequently designated peripheral myelin protein 22 which was associated predominantly with myelinating Schwann cells of the peripheral nervous system.

Comparison of the two species showed that the two transcripts were identical throughout the coding sequence, however the 5' untranslated regions were seen to vary markedly. Detailed characterisation of the human gene identified two putative promoter regions, P1 and P2, upstream of two alternative non-translated exons designated exon 1a and exon 1b respectively (Suter *et al.*, 1994). The alternative transcripts in turn correspond to the CD25 and SR13 species (Spreyer *et al.*, 1991; Welcher *et al.*, 1991). The conservation of this gene arrangement in the rat PMP-22 gene and the high degree of sequence homology within the non-coding exons between species indicated the importance of this region.

3.1.1.1 Expression of the Alternative Transcripts

Originally described as a protein associated with the nervous system, PMP-22 has also been found in a number of other sites. Detectable amounts of both transcripts have been found in a range of non-neural tissues including the heart, lung and intestine (Suter *et al.*, 1994). In each instance preferential expression of the exon 1b containing moiety was seen. Northern analyses of a similar group of tissues were able to identify high levels of exon 1b containing transcripts but near or below detectable levels of exon 1a mRNA (Bosse *et al.*, 1994).

In the Schwann cells of peripheral nerve however, the ratio between the transcripts was reversed, the predominant PMP-22 transcript was that of exon 1a, found at levels 7 fold higher than that of exon 1b (Bosse *et al.*, 1994).

3.1.1.2 Regulation of the PMP-22 Gene

Expression levels of the total PMP-22 mRNA in the peripheral nervous system have been shown to be regulated both during development and following nerve injury (Spreyer *et al.*, 1991; Welcher *et al.*, 1991; Snipes *et al.*, 1992; Kuhn *et al.*, 1993). The contribution of the alternate transcripts to this has been investigated.

3.1.1.2.1 Developmental Regulation

During development the amount of PMP-22 protein in the peripheral nervous system is seen to rise with the increased rates of myelination. Further resolution of this expression pattern has shown that the P1 promoter driven exon 1a transcript is strongly regulated during this time. At birth, exon 1a transcript levels account for approximately 30% of the total PMP-22 mRNA population. This steadily increases to a peak at 14 postnatal days of age, at which point exon 1a transcripts contribute up to 80% of the PMP-22 mRNA species (Bosse *et al.*, 1994; Suter *et al.*, 1994). These changes correspond to the developmental cessation of Schwann cell proliferation and the peak of myelination.

In comparison, although exon 1b transcript levels rise 7 fold following birth, levels peak at post-natal day 4 in a manner that does not correlate with the formation of myelin.

The ratio between the two transcripts in the peripheral nervous system also alters developmentally, reflecting the shift in the pattern of expression during early post-natal development. Five days after birth the ratio of exon 1a to exon 1b in a normal mouse sciatic nerve is found to be 0.2. This rises to 1.4 at day15 and subsequently to 2 at day 25, as exon 1a levels exceed those of 1b (Garbay *et al.*, 1995).

3.1.1.2.2 Regulation Following Nerve Injury

Differential expression of the alternative transcripts is also seen following nerve injury. Northern analysis of the behaviour of the mRNA species following nerve crush shows the immediate decrease in both transcripts with very low levels detected 2 days after the lesion. Subsequently, the exon 1b transcript returns to control levels within one week post crush whilst exon 1a recovery begins in the second week and approaches normal levels at approximately 6 weeks after the initial lesion (Bosse *et al.*, 1994). The pattern of expression for exon 1a PMP-22 transcripts closely resembles that of other myelin proteins following injury (Gupta *et al.*, 1988; Kuhn *et al.*, 1993).

3.1.1.3 Promoter Structure

Much of the work in dissecting the roles of each of the alternative promoters and their non-translated exons has been carried out by Suter *et al.* (1994). Using DNA sequencing, primer extension and RNase protection assays they were able to map the promoter and exon regions in the human *PMP-22* gene. Analysis of the resulting sequence identified a number of regulatory components within each promoter that implicate possible roles for each.

Elements associated with the regulation of tissue specific genes have been found within the P1 promoter. 30 base pairs upstream of the mRNA cap site lies a TATA like sequence and at position –43 to –47 an inverted CCAAT box is found. The position of these motifs remains constant in the mouse PMP-22 P1 promoter sequence. These regions are absent from the P2 promoter. The P2 regulatory region contains many G and C nucleotides. A section spanning -127 to 130bp of the sequence is identified as a potential CpG island (Van de Weetering *et al.*, 1999), these are often associated with the promoters of house keeping genes (Larsen *et al.*, 1992; Cross and Bird, 1995).

A number of recognition sites for various transcription factors can be found in each of the human promoters. The transcription factor NF1 has two binding sites in P1, -217 to-221 and –241 to –245 (Zhang and Miskimins, 1993). Overlapping consensus sequences for AP-2 and Sp-1 are present at –67 to –79 in P2 (Imagawa *et al.*, 1987).

Sequence comparisons were carried out against the promoter regions of other myelin proteins. A number of elements of the myelin basic protein (MBP) promoter were also found in P1 and P2. A motif downstream of the TATA sequence in promoter P1 corresponds closely to a MBP enhancer element (Fors *et al.*, 1993). In the mouse MBP gene this is found to be involved in the binding of the transcription factor NF1 (Tamura *et al.*, 1988; Aoyama *et al.*, 1990). In addition, a similar sequence to that of the M1 binding site of the MBP promoter (Aoyama *et al.*, 1990) is found in the P2 sequence.

Within the P1 promoter is a region that shares homology with a range of myelin protein promoters. The FP330 sequence identified in the proteolipid protein (PLP) promoter is also present in the protein zero (P_O) promoter of rat, mouse and human. It can also be found in MOTIF B of the MBP promoter (Nave and Lemke, 1991) and corresponds to bases –78 to –67 of the human P1 promoter. Analysis of the murine PMP-22 P1 promoter sequence identifies each of these sites within the mouse promoter (Table 3.1.1.3).

Functional studies of the rat PMP-22 promoters (Suter *et al.*, 1994) have confirmed that both regions can confer promoter activity. 3kb of the upstream region of each non-translated exon were cloned into a plasmid vector containing the gene for bacterial chloramphenicol acetyltransferase (CAT) in both the forward and reverse orientation. The resulting plasmids were then transfected into primary Schwann cells and the hamster fibroblast line RJK88. The plasmid containing the forward orientation of the P2 promoter proved to be the most active in driving CAT expression, whilst P1 promoter activity was much lower, however the structure of this region is still thought to suggest that it performs a regulatory role.

The P1 promoter has therefore been established as the Schwann cell specific promoter for the expression of PMP-22, a protein involved in the maintenance of the myelin sheath. PMP-22 is expressed as one of the early myelin proteins concomitant with P_O but before MBP (Notterpek *et al.*, 1999). Its use to drive the expression of a reporter gene should therefore give rise to the Schwann cell specific expression of that gene.

Mouse	MBP	Enhancer	-127	AACTGGCAAGGCGCCCACCCAG -106
Human	PMP-22	Promoter 1	-27	**AACTGGAAAGACGCC**TGGTC**TG** -6
Mouse	PMP-22	Promoter 1	-27	**AACTGGAAAGACGCC**TAGGACA -6
Human	PLP	FP330	-341	GGGCTCTCACTT -330
Human	P_0	Promoter		**GGGCTGTGAGGC**
Rat	P_0	Promoter	-367	**GGGCTCTCA**GGC -356
Mouse	P_0	Promoter		**GGGCTCTCA**GGC
Mouse	MBP	Motif B	-455	**GGGCTCTCA**GGC -444
Human	PMP-22	Promoter 1	-78	AAC**CTCTCA**GGC -67
Mouse	PMP-22	Promoter 1	-78	AAC**CTCTCA**GGC-67

Table 3.1.1.3 Comparison of conserved sequences in the promoters of myelin genes. Highlighted bases are conserved between motifs. Underlined sequences refer to NF1 binding sites.

3.1.2 The Reporter Gene – Green Fluorescent Protein

Reporter genes are commonly used to assess gene expression and regulation (Alam and Cook, 1990). Applications include the study of promoter and enhancer elements. Reporter genes can be useful in normalising transfection reactions, or in the production of stable cell lines with selectable markers. Reporter genes have also been used to create transgenic mice in which cells expressing specific genes can be identified.

Two of the most frequently used reporter genes are chloroamphenicol acetyltransferase (CAT) and the *lac Z* gene for β-galactosidase. When engineered into plasmids in which their expression is driven by a designated promoter factors that regulate the promoter and its use can be assessed by the changes in reporter gene expression. These enzymes catalyse reactions that can then be quantified by the amount of reaction products produced. To do this, substrates have to be provided and the tissue fixed (Silhavy *et al.*, 1972; Gorman *et al.*, 1982; Sanes *et al.*, 1986; Ginot *et al.*, 1989).

Recently, an alternative reporter gene has been described, the advantages of which include the lack of any need for substrates and co-factors. It can be assessed in live cells, with no need for fixing and staining protocols and is ideal for studying the dynamic movements of cells. This reporter gene is the green fluorescent protein.

The Pacific Northwest jellyfish *Aequorea victoria* is seen to fluoresce in response to excitation with blue light. This process is mediated by a photoprotein, the aequorin species. Once activated by calcium binding (Shimomura *et al.*, 1962) the aequorin in turn excites the green fluorescent protein (GFP, Morin and Hastings, 1971; Morise *et al.*, 1974).

Characterisation of the GFP protein has elucidated an extremely useful tool. The gene was cloned by Prasher *et al.* (1992) and Chalfie *et al.* (1994), it consists of 238 amino acids and is 30 kDa in size. The chromophore is extremely stable to a range of stresses. Fluorescence remains in temperatures up to 65°C, pH 11, in solutions of 1% SDS and in the presence of 6M guanidinium chloride. It can also resist many proteases for a number of hours. A loss of fluorescence due to acid or base treatment or the presence of a denaturing agent can be recovered by neutralisation of the pH or removal of the agent (Chalfie *et al.*, 1994).

GFP absorbs blue light at 395nm wavelength with a smaller peak at 475nm, these peaks emit green light at 509nm and 540nm respectively (Morin and Hastings, 1971; Morise *et al.*, 1974; Ward *et al.*, 1980). The protein has a tendency to dimerise and it is the dimerised form that is able to interact with the primary photoprotein, aequorin, and that is predominant in fluorescent organelles of the jellyfish (Cubitt *et al.*, 1995). The chromophore structure lies at the 3' end of exon II of the GFP gene (Prasher *et al*, 1992). It is formed by the cyclisation of a tri-nucleotide sequence also found in the chromophore of the *Renilla* GFP (Prasher *et al.*, 1992; San Pietro *et al.*, 1993). Cyclisation of Ser65, Tyr66 and Gly67, with 1,2-dehydrogenation of the tyrosine residue creates the fluorescent species (Prasher *et al.*, 1992; Cody *et al.*, 1993).

3.1.2.1 Mutations in the Green Fluorescent Protein

Mutations of the wild type protein to dissect the mechanism of fluorescence and to determine more advantageous forms have been extensive. Many mutations of the GFP protein result in the abolition of the chromophore or reduced fluorescence. Replacement of the Ser residue at position 65 with Thr, Lys, Leu, Val or Ala gives rise to species with a single absorption peak, the 395nm peak is lost and the 475nm peak has been shifted to longer wavelengths (Heim *et al.*, 1995). One of these, the Ser65Thr mutation gives rise to a protein with a number of benefits. It has an excitation peak of 490nm and emits at 510nm. It has six fold greater brightness than the wild type protein, and it oxidises to the final fluorescent species at a four fold faster rate than the wild type (Heim *et al.*, 1995). The mutated protein does not undergo photoisomerisation and photobleaching occurs more slowly (Cubitt *et al*, 1995). This makes this GFP mutant particularly suitable for use as a fluorescent marker.

A number of commercial variations of GFP are available, each tailored to different applications. The Clontech plasmid pEGFP-1 (Genbank accession number U55761) contains a red shift variant of the wild type GFP which shows an excitation maximum of 488nm and emission maximum of 507nm. The enhanced form of GFP (EGFP) contains two mutations, the Ser 65 Thr mutation and substitution of Phe 64 to Leu. The coding sequence contains more than 190 silent base changes corresponding to human codon usage preferences (Haas *et al.*, 1996). Kozak consensus transcription initiation sites and downstream polyadenylation signals ensure correct processing of the protein (Kozak, 1987).

The pEGFP-1 vector lacks a promoter sequence, allowing it to be used to assess the activity of promoters and enhancer elements inserted into the multi-cloning site (MCS).

3.1.2.2 Expression of the Green Fluorescent Protein

The green fluorescent protein has been successfully expressed in a number of systems. Both prokaryotic and eukaryotic cells are seen to fluoresce with GFP expression indicating that its chromophore formation is species independent and does not require interaction with other gene products. The fluorescence must occur

through the use of ubiquitous cellular components or autocatalysis (Chalfie *et al.*, 1994).

GFP has been expressed in *E. coli* and *C. elegans* (Chalfie *et al.*, 1994) as well as many plant species (Sheen *et al.*, 1995; Hu and Cheng, 1995). *Drosophila melanogaster* and zebrafish also successfully express GFP (Wang and Hazelrigg, 1994; Peters *et al.*, 1995; Amsterdam *et al.*, 1995 as cited in Zhou *et al.*, 1997).

The expression of GFP in mammalian cells and as a reporter gene in transgenic animals has been reported, and these studies indicate some of the advantages of GFP as a fluorescent marker.

Ikawa *et al* (1995a,b) have used the ubiquitous β-actin promoter to drive GFP expression in transgenic mice and were able to show that in these mice, embryos at the morula stage of development were already displaying GFP fluorescence. A similar study was repeated elsewhere with equivalent results (Takada *et al.*, 1997; Okabe *et al.* 1997).

The tagging of GFP to a protein can provide detailed information about the dynamic interactions of that protein. For example a fusion construct of the glucocorticoid receptor with GFP allowed confocal time lapse microscopy to show the hormone dependant translocation of the receptor from the cytoplasm to the nucleus (Htun *et al.*, 1996).

Tissue specific direction of GFP expression has also been demonstrated. Glial fibrillary acidic protein (GFAP) is an intermediate filament used to identify astrocytes. The promoter region of GFAP was coupled to the GFP gene and was used to create a transgenic mouse (Zhou *et al.*, 1997). The astrocytes in multiple regions of the brain were positive for GFP expression, providing a system in which dynamic changes in astrocyte morphology during development and injury can be followed.

3.1.3 Defining the Transgene Construct

Having identified a suitable myelin protein promoter with the desired expression pattern and a reporter gene that will give strong fluorescence and requires no manipulation or co-factors a strategy was determined to produce a plasmid vector containing these constituent parts that could be used to produce a transgenic mouse.

The P1 promoter of PMP-22 was to be isolated and used to drive the expression of EGFP in a temporal and tissue specific manner in a transgenic mouse line.

3.1.3.1 Production of the Transgenic Construct: A Summary of Procedures

Isolation of the P1 Promoter
Primers were designed to the known sequence of the promoter. This was extracted by PCR from mouse genomic DNA.

⇩

Ligation into Intermediate Vector.
The T overhang in pGEM T easy vector (Promega) enables ease of ligation of the PCR product.
Restriction digest and DNA sequencing confirms the identity of the fragment.

⇩

Production of the Construct.
The P1 region was excised and inserted into the MCS of pEGFP-1 vector (Clontech).
Successful ligation was assessed by restriction digest

⇩

Expression Studies.
Transfection experiments were used to determine the functional expression of the construct in a range of cell types.

⇩

DNA Preparation
Preparation of the DNA to a quality for use in microinjection.

3.2 Results

3.2.1 Optimising the PCR Conditions

Primers were designed against the published sequence from the NCBI Genbank data base, accession number AJ001035. This sequence was first submitted by Bosse *et al.* in 1994 and later published by the same group (Bosse *et al.*, 1999). Primers were determined according to the following set of criteria. They must incorporate the full length of the required sequence and be of at least 18 base pairs in length. This ensures the high specificity of the primer to the template DNA. Since G/C and A/T binding occurs with different strengths, primers with equal numbers of both will ensure it binds with sufficient tenacity. For ease of the reaction, each of the two primers should anneal to the template at approximately the same temperature. Importantly primers were designed to prevent the formation of internal secondary structure that could inhibit binding of the primer to the desired sequence within the genomic DNA.

Conventional polymerase chain reaction (PCR) techniques utilising a single optimum annealing temperature proved unsuccessful in isolating the P1 promoter. In an attempt to extract the regulatory region from the mouse genomic DNA alternative protocols were adopted.

Hot start PCR aims to prevent non-specific binding of primers at lower temperatures and so eliminate non-specific reaction products (Chou *et al.*,1992). By separating the reacting components either physically (Chou *et al.*, 1992, Horton *et al.*, 1994) or through enzyme modification (Kellogg *et al.*, 1994, Birch *et al.*, 1996) until the reaction temperature approaches that of the annealing temperature, non-specific binding is prohibited. Further advantage is given to specific priming when a touchdown PCR protocol is observed (Don *et al.*, 1991; Hecker and Roux, 1996). In reducing the annealing temperature during subsequent rounds of amplification towards an optimum, it is hoped that less stable, non-specific binding that usually occurs at lower temperatures could be eliminated.

3.2.2 Isolating the PMP-22 P1 Promoter

For a single 100µl PCR reaction the following components were added to a 0.25ml sterile eppendorf tube maintained on ice:

1x Taq DNA polymerase buffer (10mM Tris pH 8.3, 50mM KCl, 0.1mg/ml gelatin)
dNTP's 0.2mM (dATP/ dTTP/ dCTP/ dGTP)
0.5 – 2.5 mM $MgCl_2$
100-250ng genomic DNA
Forward and reverse primers at a concentration of 1µM
Sterile H_2O to a final volume of 100µl

Oligo	Sequence 5' to 3'	Position in Promoter P1
Forward	AGA ACT AGG GCC TCG AAC T	-722 to –704
Reverse	GCC CAC AGC AGA CCA GAC T	62 to 80

Table 3.2.2.1 Oligonucleotide primers directed against the PMP-22 P1 promoter region.

A number of duplicate reactions were carried out to optimise the $MgCl_2$ concentration. The reaction was spun briefly to mix the components before the addition of 100µl of light mineral oil. The tubes were placed in a Techne PCH-3 thermal cycler and the first round of denaturation and annealing was performed. 1.0 unit of Taq DNA polymerase was then introduced to each reaction and the amplification step allowed to proceed. Cycling parameters were as follows:

		Temp (°C)	Time (seconds)
Two cycles:	denaturation	92°C	60
	annealing	68°C	45
	extension	72°C	45
Two cycles:	denaturation	92°	45
	annealing	67°C	45
	extension	72°C	45

The annealing temperature was reduced by 1°C every second cycle until the optimum annealing temperature of 58°C was reached.

		Temp (°C)	Time (seconds)
Fifteen cycles:	denaturation	92°C	45
	annealing	58°C	45
	extension	72°C	45
One cycle:	denaturation	92°C	45
	annealing	58°C	45
	extension	72°C	120

Table 3.2.2.2 Touchdown PCR conditions for the isolation of the P1 promoter from genomic DNA.

Control reactions were performed in tandem to monitor contamination by foreign DNAs. These consisted of reactions with no template DNA, no enzyme and no primers.

3.2.3 Identification of the Reaction Product

An aliquot of each PCR reaction was separated on a 1.2% agarose gel by electrophoresis (see section 2.5.3). A reaction product at the expected molecular weight of 0.8 kb was found and potential identification of the fragment as the P1 PMP-22 promoter was achieved by restriction digest (Table 3.2.3.1 and Figure 3.2.3.2).

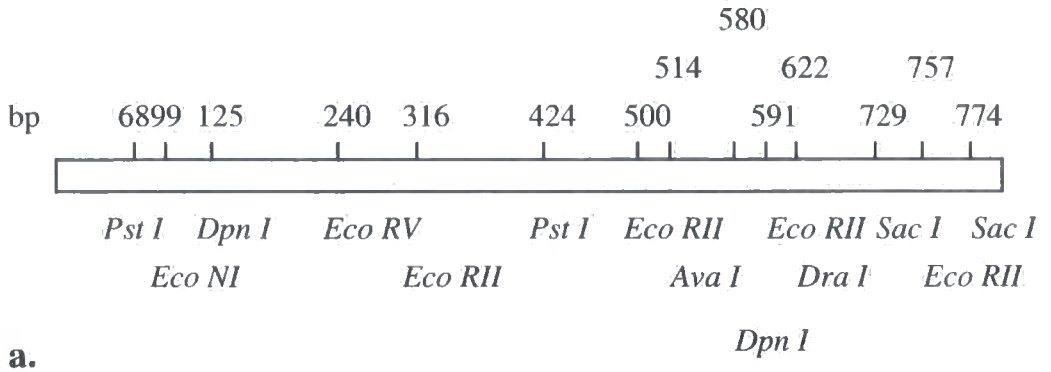
A second aliquot of the PCR reaction was run in a low melting point agarose gel, at 1.2%, the 0.8kb band was excised and the DNA extracted from the gel following the protocol described in section 2.5.3.1.

Enzyme	Restriction Site	Fragment Length
Ava I	514	514, 288
Dpn I	125, 580	125, 455, 222
Dra I	622	622, 140
Eco NI	99	99, 703
Eco RII	316, 500, 591, 757	316, 184, 91, 166, 45
Eco RV	240	240, 562
Sac I	729, 774	729, 45, 28

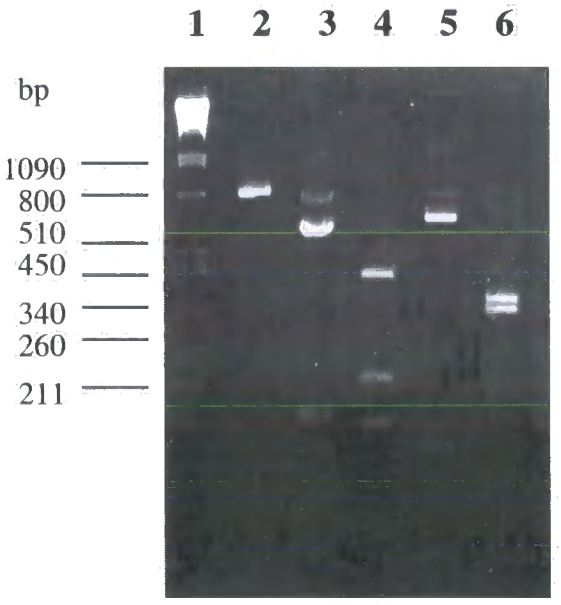
Table 3.2.3.1 Identification of the 0.8kb PCR product by restriction digest. The table shows the restriction sites and the resulting fragment lengths of a number of common enzymes.

3.2.4 Cloning of the Putative promoter into an Intermediate Plasmid Vector

The 0.8 kb DNA fragment was then ligated into the pGEM T-easy vector (Promega) at 4°C overnight as described in section 2.5.6. This vector had been commercially prepared with thymine overhangs to allow straightforward ligation of PCR products in which the Taq polymerase has added 3' adenine residues. The ligation was electrotransformed into bacteria (section 2.5.8.2). Using the blue white selection



a.



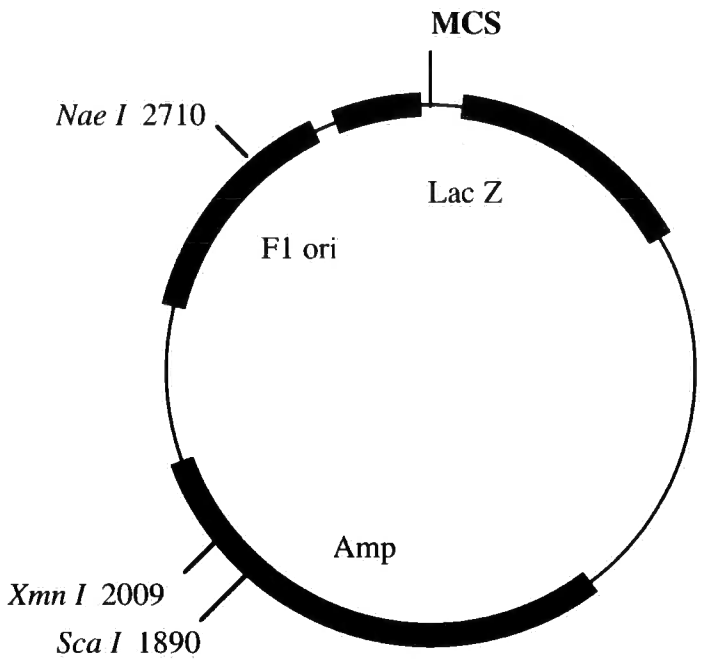
b.

Figure 3.2.3.2 Analysis of the Putative P1 Promoter
a. Schematic demonstrating restriction sites within the P1 promoter sequence. **b.** Restriction digest of the isolated 0.8kb PCR fragment. Lane 1, molecular weight marker, lambda DNA digested with Pst I. Lane 2 the uncut 0.8kb PCR fragment. Lanes 3-6 the PCR fragment digested with Dra I, Dpn I, Eco NI and Pst I respectively.

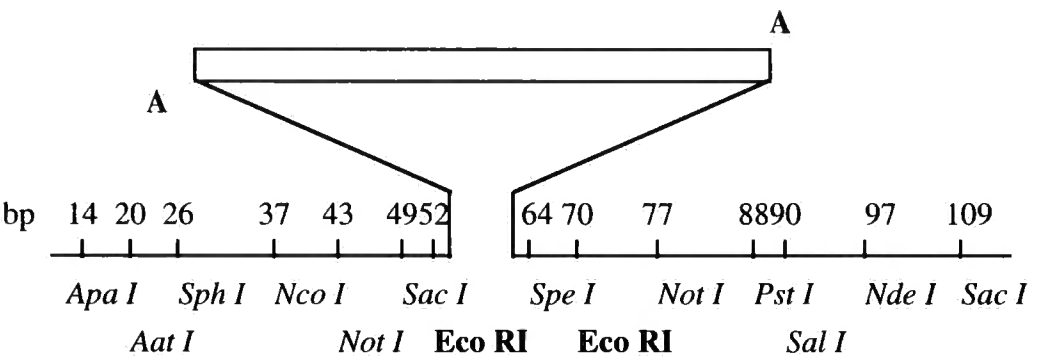
procedure (section 2.5.9) ten of the white colonies were streaked out on agar plates containing the appropriate antibiotics and grown over night. To identify clones containing the promoter insert a small amount of each resulting colony was introduced into the PCR reaction as a source of DNA. One positive colony was identified directly by PCR using the above strategy (section 3.2.2.2). DNA was extracted from this colony by the miniprep method (section 2.2.1) and enzymatic digest established the successful integration of the 0.8kb fragment (Table 3.2.4.1 and Figure 3.2.4.2). DNA sequencing with the universal primers T7 and SP6 (section 2.6) confirmed this fragment as the PMP-22 P1 promoter region.

Enzyme	Restriction Site	Fragment Length
Forward Insertion		
Dra I	682, 2078, 2097, 2789	1396, 19, 692, 1713
Eco NI	159	linearise 3820
Eco RI	52, 872	820, 3000
Pst I	28, 484, 890,	356, 406, 3058
Sac I	789, 834, 911,	45, 77, 3689
Reverse Insertion		
Dra I	240,2078, 2097, 2789	1859, 41, 651, 1269
Eco NI	763	linearise 3820
Eco RI	52, 872	820, 3000
Pst I	438 794, 890	356, 96, 3368
Sac I	88, 133, 911	45, 778, 2995

Table 3.2.4.1 Restriction sites within the pGEM T-easy/PMP22 P1 promoter plasmid. Details are given for the integration of the fragment within the plasmid in both the forward or reverse orientation.

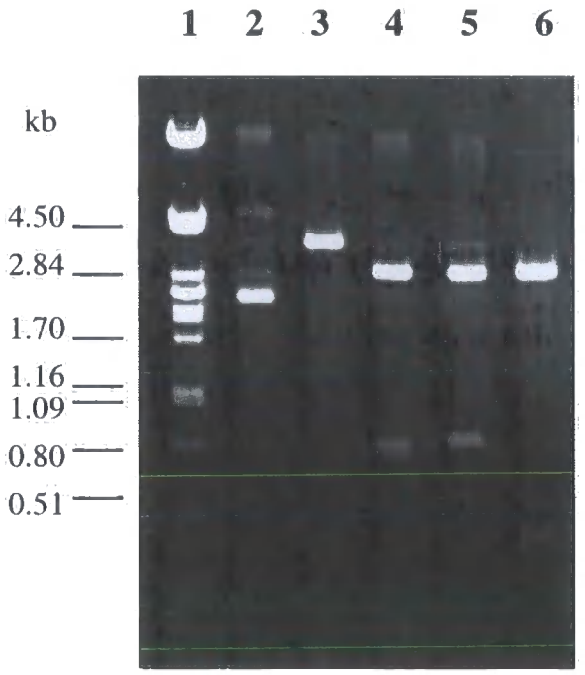


a.



b.

Figure 3.2.4.2 Analysis of the Intermediate Plasmid pGEM-P1
a. Promega pGEM-T easy vector (3kb) contains T residue overhangs for ligation of PCR products. **b.** The 0.8kb P1 PCR product was ligated into the insertion site. The fragment is later easily removed by digestion with flanking enzymes from within the multi-cloning site (MCS).



c.

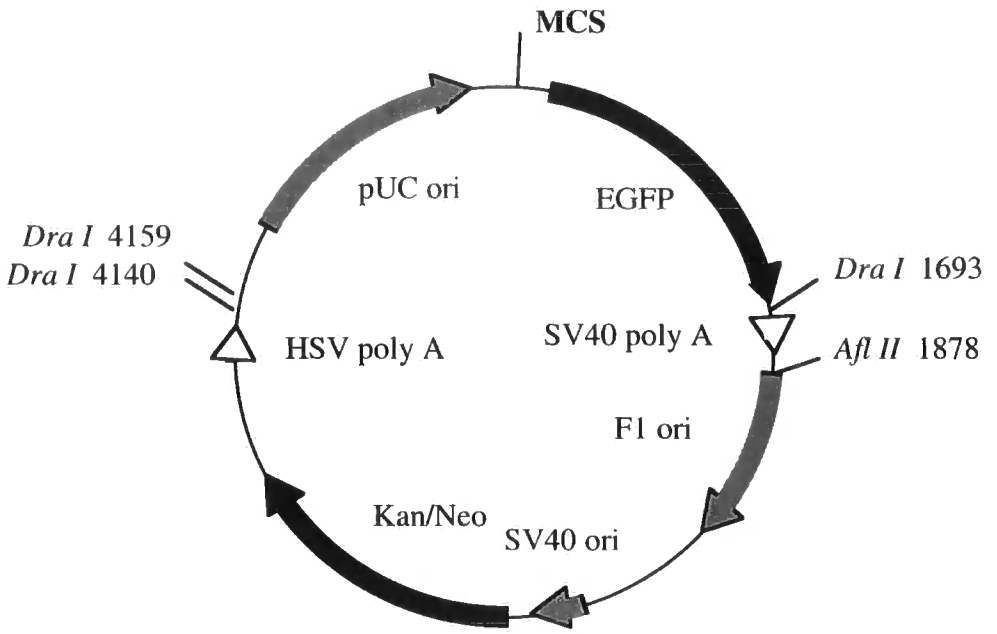
Fig 3.2.4.2c Restriction digest of the intermediate plasmid
Digestion of the P1-pGEM plasmid. Lane 1 molecular weight marker. Lane 2 uncut intermediate plasmid DNA. Lane 3-6 plasmid DNA restricted with Eco NI, Eco RI, Not I, and Pst I respectively.

3.2.5 Producing the Promoter-EGFP Construct

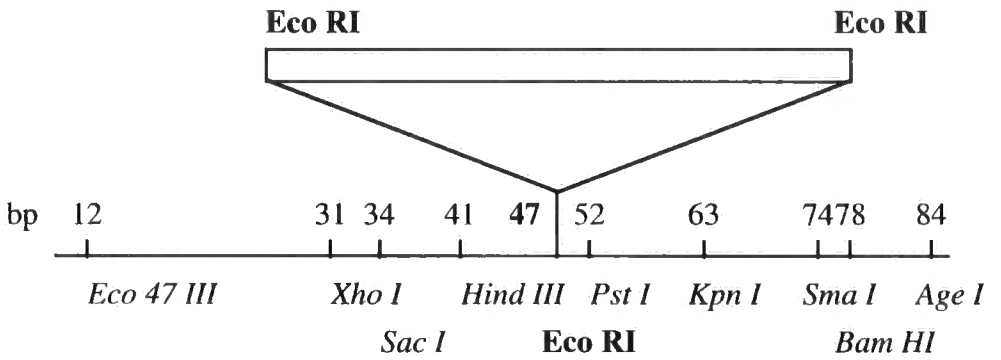
The promoterless pEGFP-1 vector (Clontech) was linearised through the multi-cloning site with the restriction enzyme Eco RI. Concomitantly the PMP-22 P1 gene was removed from the pGEM T-easy backbone by digestion with Eco RI. The restriction products from both reactions were run on a 0.8% agarose gel and the desired fragments from each extracted using the protocol described in section 2.5.3.1. Following dephosphorylation of the vector backbone with calf intestinal alkaline phosphatase enzyme (section 2.5.5), the compatible ends of vector and insert allowed cohesive ligation following the procedure in section 2.4.6 at 4°C overnight (section 2.5.6). The ligation reaction was transformed via the calcium chloride method into bacterial cells (section 2.5.8.1). Successful ligation was assessed by PCR as described in section 3.5.2.2. The DNA from six positive colonies was digested with Pst I and Sac I to determine the orientation of insertion. A specimen with forward insertion was then digested fully to ensure the correct fragment had been incorporated (Table 3.2.5.1 and Figure 3.2.5.2).

Enzyme	Restriction Site	Fragment Length
Forward Insertion		
Dra I	687, 1698, 4140, 4159	1020, 2442, 19, 1490
Eco NI	155	linearise at 4971
Pst I	124, 480, 877	356, 397, 4218
Sac I	39, 785, 830	746, 45, 4180
Hind III/Bam HI	41, 899	858, 4113
Hind III/Afl II	41, 1878	1837, 3134
Reverse Insertion		
Dra I	238, 1698, 4140, 4159	1460, 2442, 19, 1050
Eco NI	761	linearise 4971
Pst I	436, 29, 877	356, 85, 4530
Sac I	39, 86, 131	47, 45, 4879
Hind III/Bam HI	41, 899	858, 4113
Hind III/Afl II	41, 1878	1837, 3134

Table 3.2.5.1 Restriction table of the PMP22 P1 – EGFP plasmid. The restriction pattern of the plasmid digested in both the forward and reverse orientation of the P1 promoter into the vector backbone is considered.

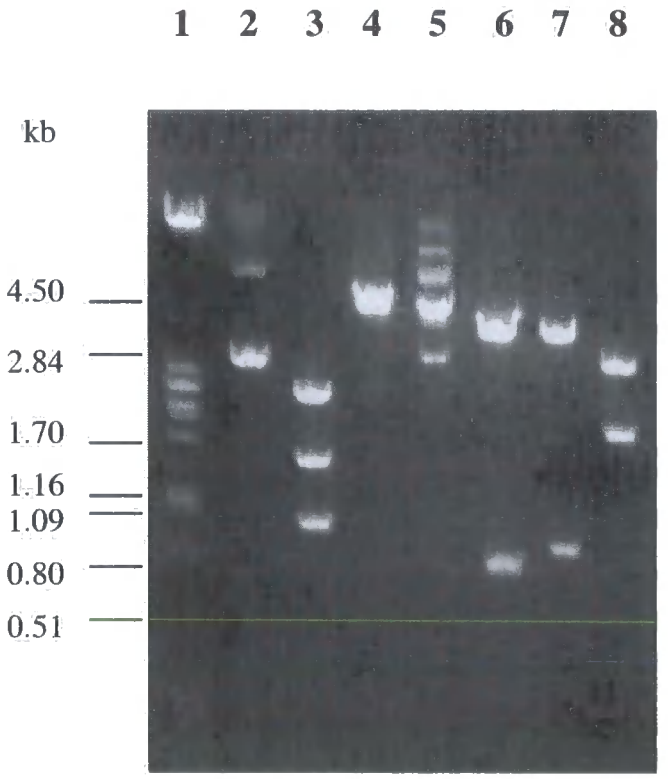


a.



b.

Figure 3.2.5.2 Production of the final P1-EGFP construct.
a. Plasmid map of the Clontech pEGFP-1 vector (4.2kb). This plasmid contains a multi-cloning site (MCS), the EGFP gene is found between base pairs (bp) 97-816 and a Kanamycin/Neomycin resistance gene. **b**. Expansion of the MCS of the pEGFP-1 vector showing the insertion of the P1 promoter into the Eco RI restriction site.



c.

Figure 3.2.5.2c Restriction digest of the final P1-EGFP construct.
Lane 1 molecular weight markers. Lane 2 uncut P1-EGFP plasmid DNA. Lanes 3-8 show restriction of the plasmid with the following enzymes Dra I, Eco NI, Pst I, Sac I, Hind III and Bam HI, Hind II and Afl II.The Hind III and Bam HI double digest releases the P1 promoter (800bp). Restriction with Hind III and Afl II excises the P1 promoter, the EGFP gene and the SV 40 poly-A tail from the plasmid (1.7 kb).

3.3 Functional Studies of the Promoter Construct

Following the successful cloning of the murine P1 PMP-22 promoter into the pEGFP (Clontech) vector backbone, functional studies were carried out. These were devised to ensure that the fragment of 5' sequence that had been isolated would prove sufficient to be capable of driving the EGFP expression and that it could do so in a manner that retained the specificity seen *in vivo*.

Previous functional studies of the human P1 promoter region utilised 3.3 kb upstream of exon 1a to drive reporter gene expression. This was shown to be sufficient to confer limited promoter activity in both rat Schwann cells and the hamster fibroblast line RJK88 (Suter *et al.*, 1994).

3.3.1 Determination of the specificity of the construct across a range of cell types

To study the potential promoter activity of the construct and to investigate the specificity of its expression, a range of transfection experiments were carried out. Each cell type was transfected with a positive control, a cytomegalo-virus promoter (CMV) driven EGFP construct (see appendix A4). The promoterless pEGFP-1 vector (Clontech) was used as a negative control, and the pP1- EGFP construct was introduced into each cell type for analysis of its expression.

3.3.1.1 Evaluation of expression of the construct in the target cell type.

The potential of the construct to express in the target cells was important to determine prior to the production of the transgenic mouse. To investigate this, the pP1-EGFP vector was transfected into Schwann cells present within the dorsal root ganglion co-culture (section 2.11.2).

In addition a line of transformed Schwann cells were transfected and assessed for the expression of EGFP. Many cell lines have been developed from the Schwann cells of newborn and adult rat peripheral nerve using a variety of methods. While many differentiate to produce myelin when presented to axons both *in vitro* and *in vivo* (Boutry *et al.*, 1992), the transformed Schwann cells lose the ability to regulate their own growth, and in some the myelinating capability falls with repeated passaging (Porter *et al.*, 1987). Haynes *et al.* (1994) attempted to develop a Schwann cell line

develop a Schwann cell line transformed in such a way as to retain as many of the characteristics of the parent Schwann cells as possible. Neonatal Schwann cells were transformed via intermittent exposure to the mitogen cholera toxin, and the cell line SCL4.1/F7 was derived from this. These cells display many desired characteristics including the potential to produce myelin, calcium dependent substratum adhesion and the negative autoregulation of growth.

3.3.1.2 Promoter directed expression of EGFP in other PMP-22 positive cells.

Initial studies of PMP-22, while showing predominant expression of the protein in the Schwann cells of peripheral nerves, also identified many sites of non-neuronal expression (Spreyer *et al.*, 1991; Welcher *et al.*, 1991; Bosse *et al.*, 1994; Suter *et al.*, 1994). In order to determine fully the expression potential of our construct, transfection into non-neuronal cells known for their expression of PMP-22 was investigated.

PMP-22 expression begins in the developing embryo and during midgestation, at 14.5 to 16.5 days post coitus, is seen in the developing muscle (Baechner *et al.*, 1995). Primary rat myoblasts were kindly donated by a colleague. (section 2.9.2). The cultured myoblasts were able to differentiate, forming myotubes following serum depletion or the addition of differentiating media (DMEM with 5% horse serum and 2mM glutamine).

3.3.1.3 Expression of PMP-22 driven EGFP in fibroblasts.

PMP-22 was originally identified as the growth arrest specific protein (GAS-3) in the fibroblast line NIH3T3 (Manfioletti *et al.*, 1990). To investigate the potential of the P1 promoter within fibroblast cells, primary human skin fibroblasts were examined for EGFP fluorescence following transfection with the construct.

3.3.1.4 Appropriate specificity of the construct in an unrelated cell type.

Up to this point from previous observations each cell type considered has the potential to express the P1 driven EGFP protein. The P1 promoter is thought to contain control regions that regulate its specificity of expression. To ensure this specificity had been maintained in the construct its expression in an unrelated control cell type was investigated.

The cell type utilised was the COS-7 cell line, derived from the African Green monkey kidney cell line CV-1. It has been transformed by an origin defective mutant of SV40 (Gluzman, 1981).

3.3.2 Transfection Reactions

A number of transfection protocols are described in the materials and methods section (section 2.7). The transfection efficiency achieved with each cell type was optimised with each different regime using the positive control DNA. The optimal method of transfection is described below for each cell type.

Primary mouse Schwann cells in dorsal root ganglia co-cultures (section 2.11.2) were transfected via the calcium phosphate method (section 2.7.1) for six hours. The transfection media was removed and the cells washed with PBS before the addition of fresh growth media.

The transformed Schwann cell line designated SCL4.1/F7 (Haynes *et al.*, 1994) was transfected using the lipophilic reagent Fugene (Boehringer-Mannheim) as described in section 2.7.2.

Prior to transfection primary myotubes were trypsinised from the culture dish and fibroblast numbers were depleted by pre-plating on a non-permissive substrate for 30 minutes. The remaining, suspended cells, were replated on 1% gelatin coated dishes. Primary myotubes in their non-differentiated state were transfected via calcium phosphate for six hours. They were then allowed to differentiate through the depletion of serum from the media before the cells were assayed for EGFP expression.

The remaining cell types, the COS-7 green monkey kidney cells and wild type primary skin fibroblasts were also transfected with the lipophilic reagent Fugene (section 2.7.2).

48 hours following transfection cells were fixed in 4% paraformaldehyde (section 2.12.2) and mounted in mowiol (Calbiochem). EGFP expression was visualised on a BIO-RAD Microradiance Confocal microscope.

3.3.3 Results

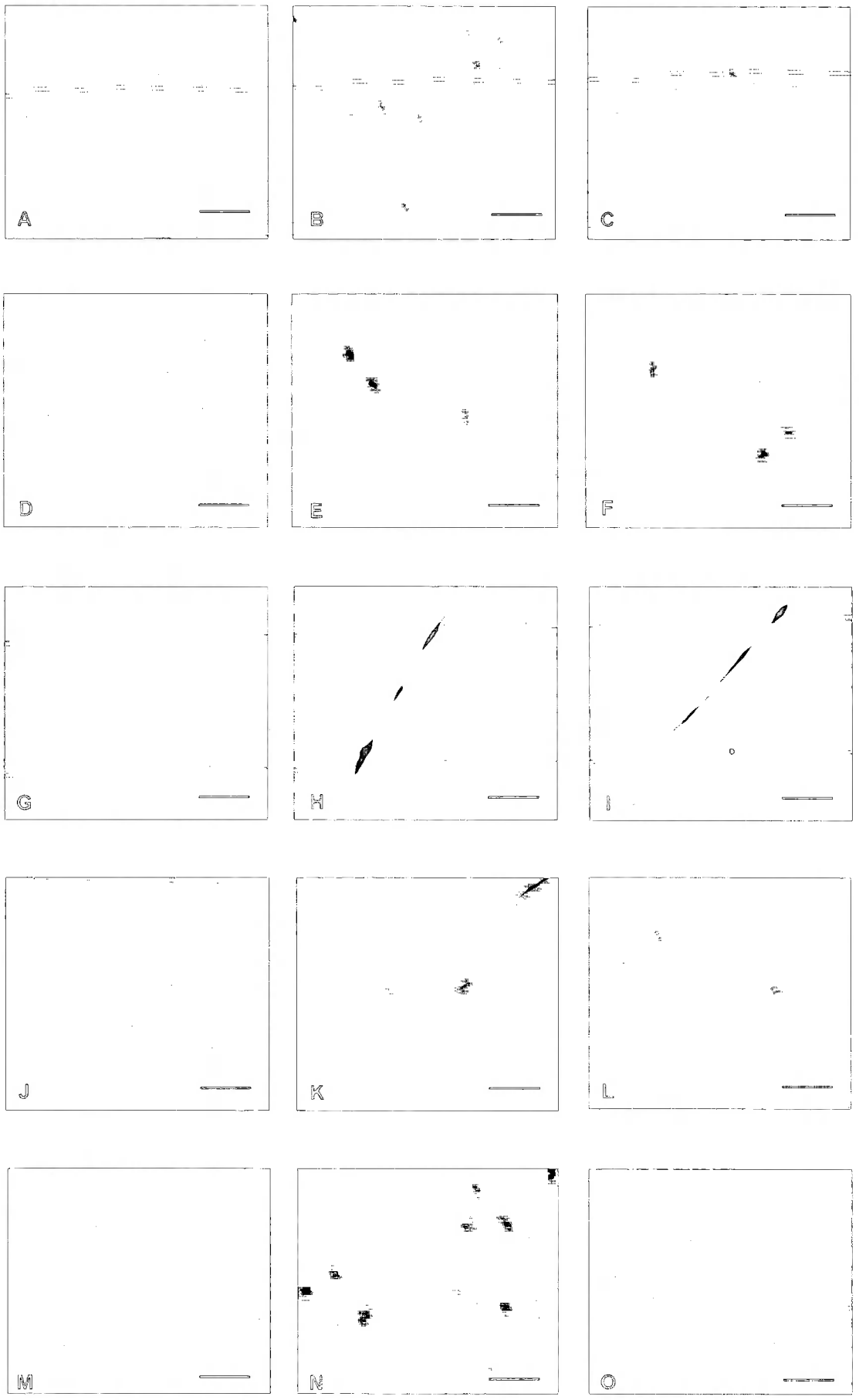
All cell types were seen to express the positive control plasmid pCMV-EGFP following their specific transfection regime (Figure 3.3.3). The pP1-EGFP construct was successfully expressed in both primary Schwann cells and the transformed Schwann cell line. In addition P1 driven EGFP expression was seen in both the primary myoblasts and primary fibroblasts. The control cell line, the COS-7 cells, did not express the construct as monitored by EGFP fluorescence.

The transfection efficiencies of each cell type were assessed approximately. The primary cell types, Schwann cells, fibroblasts and myoblasts expressed the pCMV-EGFP construct in approximately 1-2% of cells. The transformed Schwann cells and COS-7 cells expressed the positive control plasmid with approximately 5% efficiency. In all cases the P1 promoter driven expression of EGFP was less than that of the positive control.

In all cell types the promoterless expression vector, pEGFP-1 (Clontech) failed to elicit GFP fluorescence.

Figure 3.3.3 Functional studies of the pP1-EGFP construct.

A. Dorsal root ganglia co-cultures were transfected with the negative control vector pEGFP-1 promoterless vector. **B.** EGFP fluorescence in cells transfected with the positive control plasmid pCMV-EGFP demonstrated the successful introduction of the DNA. **C.** Transfection with the pP1-EGFP vector showed expression of the construct in a population of the cells in the DRG co-culture system. The Schwann cell line, SCL4.1/F7 was assessed for its ability to express the constructs **D.** SCL4.1/F7 cells transfected with the negative control plasmid, pEGFP-1 promoterless control vector. **E.** SCL4.1/F7 cells expressed the positive control plasmid pCMV-EGFP. **F.** The Schwann cell line was also able to express the pP1-EGFP construct. **G.** Primary myoblasts were transfected with the negative control plasmid, **H.** the positive control plasmid and **I.** the pP1-EGFP construct. **J.** In addition primary skin fibroblasts were assessed for EGFP expression with the negative control plasmid, **K.** the positive control plasmid and **L.** the pP1-EGFP construct. **M.** The control cell type, COS-7 cells, were transfected with the negative control plasmid pEGFP-1 promoterless vector, **N.** the pCMV-EGFP positive control vector and **O.** the pP1-EGFP construct. In all instances no fluorescence was seen in cells transfected with the negative control, and each cell type demonstrated EGFP fluorescence from the positive control plasmid pCMV-EGFP. Scale bar 100μm.


A
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3.4 Discussion

The literature provides strong evidence that the P1 promoter of the PMP-22 gene is responsible for expression of the protein in peripheral nerve, specifically within the Schwann cells. Successful isolation of the P1 promoter from murine genomic DNA with primers designed to the published sequence, has been achieved and confirmed by restriction digest and DNA sequencing. The incorporation of the P1 promoter into the Clontech pEGFP-1 promoterless vector resulted in a construct with the potential to give rise to green fluorescent protein expression in a Schwann cell specific manner. The specificity of the promoter P1, exon 1a transcript expression seen *in vivo* is maintained *in vitro* with this construct. In a series of transfection experiments reporter gene expression was seen in the target cell type, both primary and transformed Schwann cells. In addition, expression of EGFP was observed in non-neural cell types associated with PMP-22 expression, primary fibroblasts and myotubes. However, expression was not seen in an unrelated cell type, the COS-7 cell.

These data provided evidence that the isolated region did, as intended, confer promoter activity and retained specificity, and that it was suitable for microinjection with the aim of producing a transgenic line in which the reporter gene, EGFP is expressed in a Schwann cell specific manner. It could also be used to examine sites of non-neural PMP-22 expression for evidence of EGFP fluorescence.

Despite the success of the pP1-EGFP construct in maintaining specificity *in vitro* other factors may influence the expression of the endogenous PMP-22 gene *in vivo*. It is possible that other regulatory regions exist further 5' and 3' of the PMP-22 gene than is incorporated here that control the pattern of expression. In addition the transgene construct does not contain the full length of the untranslated exon 1a. The untranslated exons are suggested as having an important regulatory role due to the conservation of their sequence between species.

Chapter 4.

Production of a Transgenic Mouse

Chapter 4: The Production of a Transgenic Mouse

4.1 Introduction

The use of transgenic animals has been widespread and has been applied to a range of fields. It is commonly used to induce mutant phenotypes and to recover genetic defects. The expression of tissue specific reporter genes has allowed the study of the temporal and spatial expression pattern of proteins. A number of techniques exist to achieve insertion of foreign DNA into the genome of a mouse. Microinjection of DNA into the pronuclei of fertilised embryos relies upon non-homologous recombination of the transgene into the host genome at the single cell stage. If incorporation takes place after cell division has begun the resulting transgenic founder will be mosiac and not every cell will contain the transgene. This method is advantageous over other methods such as adenoviral infection that can result in higher frequencies of multiple insertion site and mosaicism.

The incorporation of DNA constructs into embryonic stem cells is a common method for producing chimeric mice. Electroporation is used to introduce the DNA into the cells that are then microinjected into a host blastocyst. The resulting transgenic mice will be chimeric and can be bred on to produce heterozygotes for the transgene. Embryonic stem cells allow targeted mutations that incorporate selective genes to be screened for successful integration prior to the injection into the blastocyst and in this way can increase the efficiency of success.

4.1.1 The Preparation of DNA for Microinjection.

The preparation of DNA to be used for microinjection, can have a great influence on the success of integration of the transgene. Prokaryotic vector sequences have been found to be inhibitory to transgene expression (Chada *et al.*, 1985; Krumlauf *et al.*, 1985; Townes *et al.*, 1985) and it is therefore necessary to remove as much of this sequence as possible from the DNA before microinjection. In addition investigators have observed a 5-fold greater integration rate of foreign DNA introduced in a linear form than as supercoiled plasmid DNA. No apparent relationship between fragment length and integration frequency was found (Brinster *et al.*, 1985) in fact yeast artificial chromosomes carrying several hundred kilobases of mammalian DNA have been used to construct transgenic animals via the microinjection method (Peterson *et al.*, 1993; Schedl *et al.*, 1993).

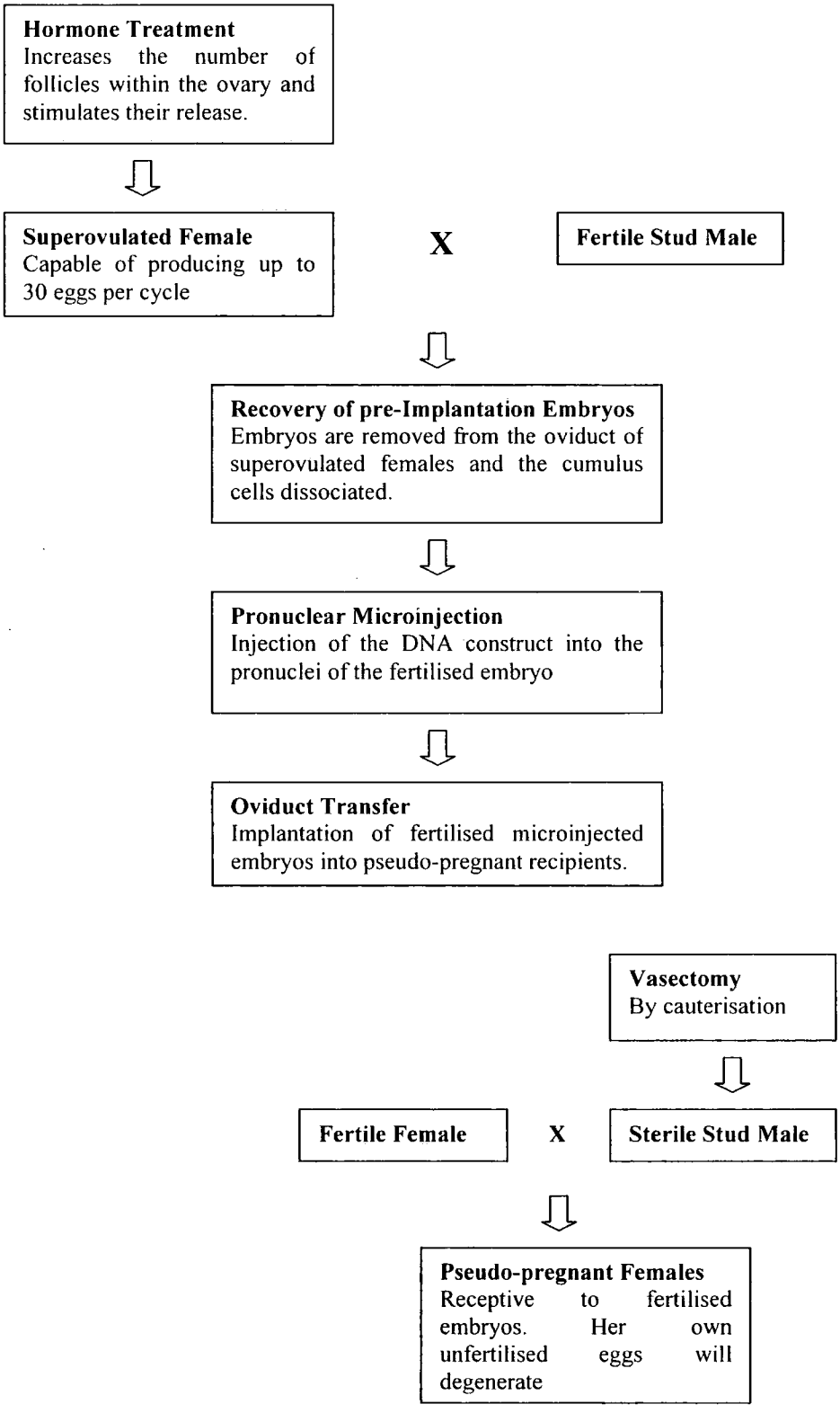
Suitably prepared DNA may integrate into the genome as a single copy, or multiple copies of the gene may insert in head to tail tandem arrays (Brinster *et al.*, 1981; Constantini & Lacy, 1981). Commonly, integration occurs at a single position, however on rare occasions multiple integration points are found (Lacy *et al.*, 1983).
The practical preparation of DNA is also crucial to the successful production of a transgenic line. The microinjection of high concentrations of foreign DNA (greater than 10μg/ml) can be toxic to the embryo, resulting in a decrease in the number of embryos developing to the morula or blastula stage in culture (Brinster *et al.*, 1985). An optimal DNA concentration range of 1-2μg/ml has been used, with no correlation found between the concentration and the eventual copy number of the transgene in founder lines. The composition of the injection buffer is also important, high levels of ethidium bromide (above 5mM) and EDTA (greater than 3mM) can also reduce the rates of embryo survival and lower integration efficiency (Brinster *et al.*, 1985).

4.1.1.1 Protocol for the preparation of DNA

PMP-22 P1-EGFP plasmid DNA was purified using Caesium Chloride and Ethidium Bromide gradients as described in section 2.2.3. The DNA was subsequently digested with the restriction enzymes Hind III and Afl II to excise the required construct from the plasmid sequence. This region includes the P1 promoter, the EGFP gene and the poly-A tail required for efficient transcription. The digest was run on a 0.8% agarose gel that did not contain ethidium bromide with appropriate molecular weight markers on either side. A section of the gel containing one lane of markers and an aliquot of the digested plasmid DNA was removed and stained in 0.25mg/ml ethidium bromide for 10 minutes to allow location of the position of the desired band under UV light. This was then repositioned alongside the remainder of the gel and the region corresponding to the required fragment excised.
The linearised DNA was recovered from the agarose gel using the Qiagen Gel Extraction kit, as described in section 2.5.3.1, and resuspended in injection buffer (10mM Tris-HCl pH 7.4 and 0.1mM EDTA pH7.4 prepared with milliQ distilled water).The concentration of DNA was determined by its spectrophotometric absorbance at 260nm wavelength, see section 2.4.1. The fragment was then identified by restriction digest. The DNA was subsequently diluted to 1.45ng/μl with injection buffer. To ensure that the DNA was free of all organic impurities it was passed through a 0.2μm Millipore filter to remove any particulate matter.

4.1.2 The Production of Transgenic Mice

A summary of procedures



4.2 Preparation of Equipment for Microinjection

The protocols described below for assembling the microinjection apparatus and the animal procedures necessary for successful microinjection are modifications of those described in Hogan *et al.* 1994.

4.2.1 The Production of pipettes for Microinjection

4.2.1.1 Handling Pipettes

Handling pipettes were used to manipulate oviducts during embryo recovery and to move embryos between stages. Medium length capillary tubes were pulled manually in the edge of a blue flame to give a fine tip of approximately 5-8cm in length with an internal diameter of 120-180μm. A diamond pencil was used to break the other end cleanly.

4.2.1.2 Transfer Pipettes

Transfer pipettes were produced as for the handling pipette and were used for transferring embryos into the infundibulum of the oviduct of the pseudo-pregnant female. Once complete, a mounting needle was heated until red hot in a bunsen flame and was then touched 1 cm from the end of the pipette. This softened the glass so that a bend could be created in the pipette tip. This was to allow it to be inserted easily into the neck of the infundibulum.

The use of both handling and transfer pipettes was controlled through attachment to an aspirator tube and mouth piece (Sigma).

4.2.1.3 Holding Pipettes

Holding pipettes were used to maintain an embryo in position during injection. Long capillary tubes were pulled on a pipette puller and a diamond pencil was used to give the tip a straight break. A microforge was used to heat the pipette until the outside tip diameter was approximately 50 μm with an inside diameter of approximately 15μm.

The holding pipette, once mounted in the apparatus was controlled by an oil driven micrometer syringe. The holding pipette itself was back filled with Fluorinert FC-77 (Sigma) as mineral oil has been reported to damage the embryos (Hogan *et al.*, 1994).

4.2.1.4 Injection Pipettes

Long capillary tubes with an internal filament were pulled on a pipette puller to give a pipette with a very fine taper to ensure the point of entry into the embryo was not so large as to cause lysis.

Before attachment to the manipulator a diamond pencil was used to break off 1cm of the bottom of the pipette. It was then inserted into a microcentrifuge tube containing DNA solution and the tip filled by capillary action with approximately 1µl.

4.3 Arrangement of the Microinjection Apparatus

4.3.1 Microscopes and Objectives

An inverted Nikon Diaphot microscope with Normarski differential interference contrast optics (D.I.C.) and x4 and x40 objectives was mounted on a base plate upon a vibration free table between two Leitz micromanipulators (Figure 4.3).

4.3.2 Control of the Holding Pipette

A Leitz instrument tube, controlled by a micromanipulator, was attached via Tygon plastic tubing to a micrometer syringe with a mechanism for fine adjustment. The entire system was filled with light paraffin oil and all air bubbles eliminated. The holding pipette was filled with Fluorinert electronic fluid FC77 using a 26 gauge fine needle inserted into the end of the pipette. This was then inserted into the instrument tube. The manipulator levels were altered to allow access to the injecting chamber.

4.3.3 Control of the Injecting Pipette

An injection pipette loaded with DNA solution was attached to a Leitz instrument tube connected via Tygon tubing to an air filled ground glass 50ml syringe. The DNA solution was expelled by pressure applied to the syringe. When the pressure was released the syringe returned to its neutral position and DNA flow stopped.

4.3.4 The Injection Chamber

A drop of M2 media (Speciality Media, Sigma) 2-4mm in diameter was placed on a glass depression slide coated with silicon (Sigmacote, Sigma). This was then covered with mineral oil to prevent dehydration. The shape of the drop is important to ensure

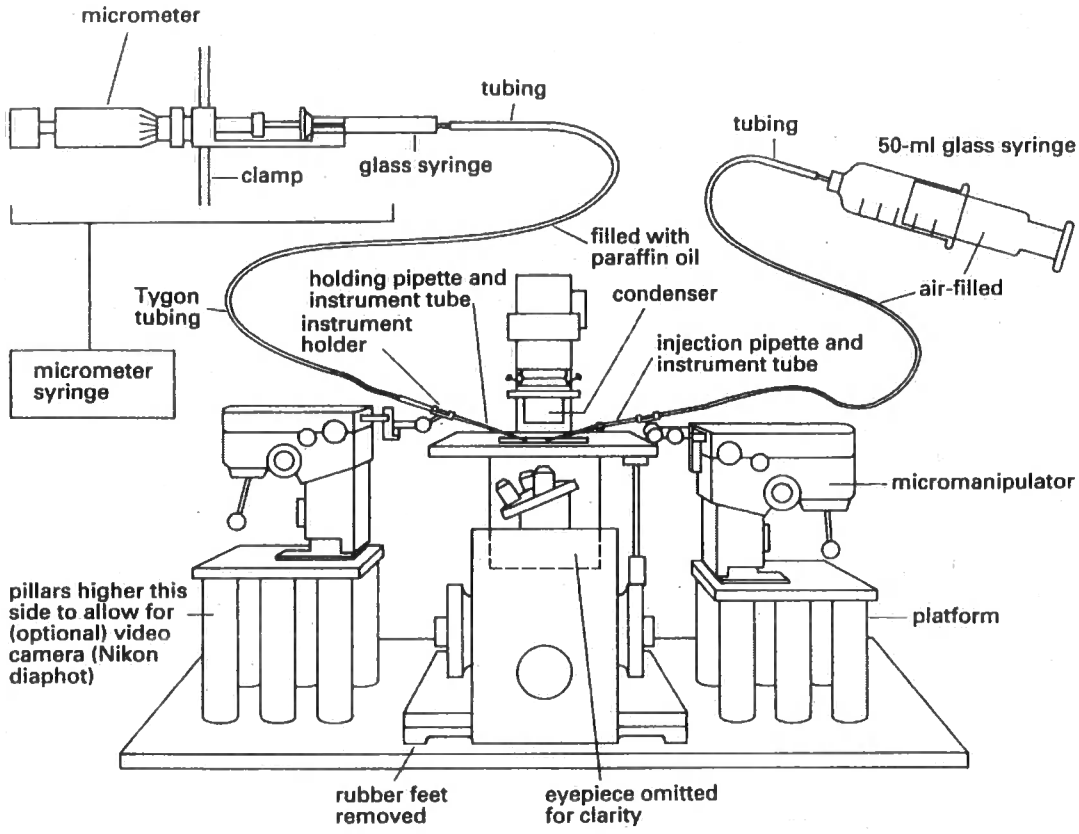


Figure 4.3 Arrangement of the microinjection apparatus
From Hogan *et al.* 1994. Arrangement of microscope, base plate and micromanipulators for pronuclear injection. The central microscope is flanked by manipulators that control the holding and injection pipette. These in turn are controlled by a micrometer syringe and an air filled glass syringe respectively that regulate the suction stabilising the embryo and expulsion of the DNA solution.

ease of access to the embryos, if it is too flat the addition of the mineral oil will depress it further. If however the bubble is too round then the embryos will slide to the sides, in either case the embryos become difficult to manipulate.

4.3.5 Correct Positioning of the Injection Chamber

To correctly position the injection chamber the media drop was centralised on the microscope stage. An M2 filled holding pipette was moved against the bottom of the slide and then upward towards the centre of the drop.

The holding pipette remained static at all times. Any necessary movement was brought about by manipulation of the microscope stage.

The loaded injection needle was positioned at a 5-10° angle from the injection chamber opposite the holding pipette and within the same plane. The fine tip of the pipette was pulled to a closed point by the puller, in order to allow expulsion of the DNA solution this point was broken by carefully sweeping it across the edge of the holding pipette. This could be repeated during injection if the tip became obstructed.

4.4 Animal Procedures Used to Produce Tansgenic Mice

4.4.1 Fertile Stud Males

Mice reach their sexual maturity at approximately 8 weeks of age and can then be used for stud for up to one year.

Studs were separated into individual cages several days before being presented to a female. This prevented fighting and avoided suppression of testosterone synthesis and sperm count in litter mates by the dominant male.

Following mating, sperm counts are reduced and so studs were rested for 2-3 days before further use. A 60-90% plugging rate among females was expected from the stud mice. Repeated failure to plug, unfertilised eggs and low sperm count were taken as indications of overuse and the mice were replaced.

4.4.2 Sterile Stud Males

Sterile stud males were used to induce pseudo-pregnancy in female recipients for oviduct transfer. Sexually mature mice were vasectomised and subsequently tested for sterility. This was denoted by observing a vaginal plug in the female that on three subsequent matings failed to result in pregnancy. A vaginal plug consists of coagulated proteins from the male's seminal fluid that dissolves within 12-14 hours following mating.

Sterile stud mice were used every other night for up to two years. Any vasectomised mouse failing to plug a female 4 to 6 times consecutively was replaced.

4.4.3 Superovulation

In order to maximise the number of embryos recovered, but to also limit the number of animals used as much as possible, a hormonal regime was adopted to increase the number of eggs produced by each female.

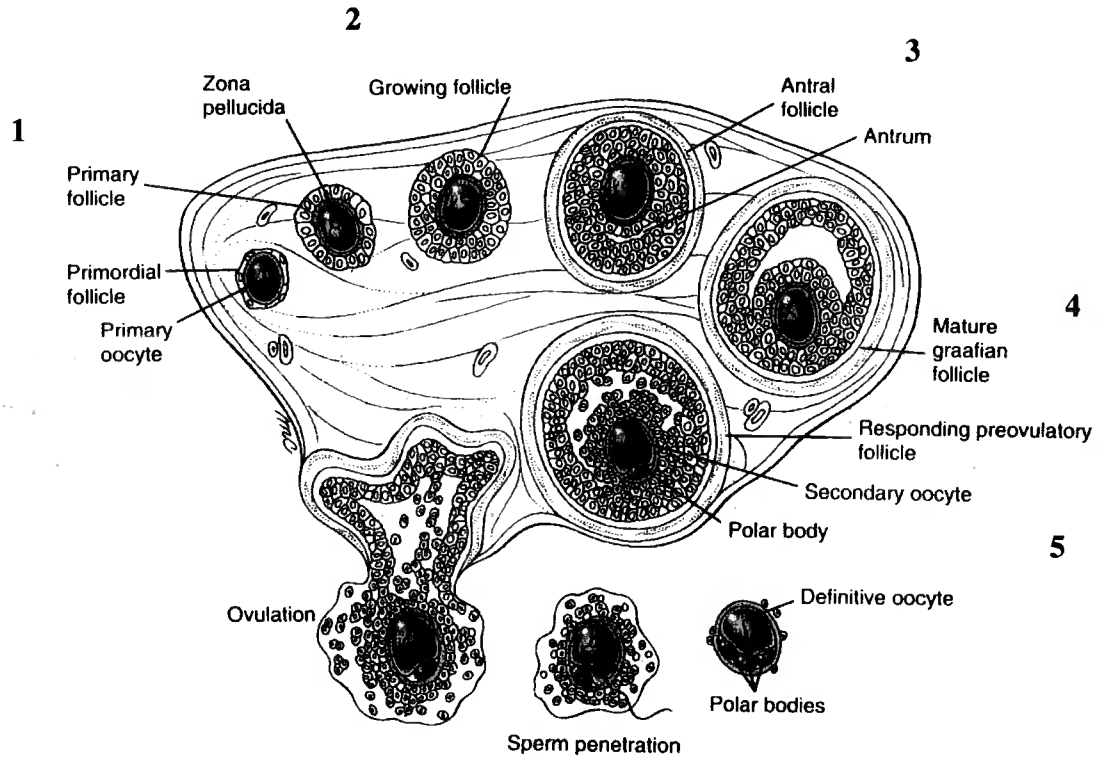
Superovulation was induced in 3 to 4 week old female mice by the administration of exogenous hormones concomitant to those naturally released. This resulted in each mouse producing between 20 and 30 eggs, two to three times the number produced in a normal cycle.

The female was first injected intraperitoneally (IP) with the commercial form of pregnant mares serum (PMS), Folligon (Intervet). Elements within this can substitute for the gonadotrophin, follicle stimulating hormone (FSH). FSH is produced by the anterior pituitary gland and acts upon those primary follicles receptive to it. The follicle cells surrounding the primary oocyte are stimulated to proliferate and form the fluid filled antrum. The low levels of lutenising hormone (LH) present at this time stimulate the production of a layer of thecal cells surrounding the follicle. These cells produce androgens, which stimulate the follicle cells to produce oestrogens. Oestrogens initiate further follicle cell proliferation giving rise to a positive feedback loop. High oestrogen levels have a second consequence; they cause the follicles to lose their sensitivity for FSH and become receptive to LH. Following this there is a surge in LH levels secreted from the anterior pituitary that causes mature follicle to be released (see figure 4.4.3).

During superovulation the amount of endogenously released LH is insufficient to cause the release of the excessive numbers of mature follicles. To aid their release a second injection of exogenous hormone is administered. The commercial form of

Figure 4.4.3 Summary of the development and hormonal control of oocyte formation. From W.J. Larsen, Human Embryology (1993).

1. The nucleus of a primary oocyte within a primordial follicle remains at prophase of division I. At this stage the follicle is approximately 20 microns in diameter. **2.** In the pre-antral follicle glycoproteins secreted by the follicle cells form the zona pellucida. The surrounding layer is penetrated by extensions of the follicle cells that connect to the oocyte via gap junctions. These links are thought to provide both developmental signals and metabolic support to the oocyte. **3.** Gonadotrophin releasing hormone from the hypothalamus stimulates the anterior pituitary to release FSH and LH. The FSH stimulates the follicle cells of receptive oocytes only, to divide and form the fluid filled antrum. The Antral follicle now spans approximately 85 microns. **4.** Stimulation of the connective tissue of the ovarian stroma by LH causes it to differentiate into the thecal layers. Thecal cells release androgens which are subsequently metabolised by the follicle cells to oestrogens which provide a number of roles. They stimulate follicle cell division, the follicle cells in turn release more oestrogens. They also stimulate the uterine mucosa to begin preparation for the implantation of fertilised embryos. There follows a surge in the levels of LH which promotes nuclear maturation and the primary oocyte completes meiosis division I to become a secondary oocyte. **5.** Sensitivity to FSH is lost and the high levels of LH cause ovulation to begin. The secondary oocyte surrounded by the cumulus cells, once ruptured from the ovary moves towards the infundibulum of the oviduct. The remaining follicle cells proliferate and become luteal cells of the corpus luteum. If at this point the female has mated the mechanical stimulation of the vaginal walls during copulation will cause the release of prolactin from the pituitary which activates the corpus luteum to produce progesterone. This inhibits the release of further FSH and LH and in doing so prevents the cycle from resuming. It also prepares the uterine tissue further for the implantation of embryos and will maintain the initial stages of pregnancy.


1
2
3
4
5
Zona pellucida
Growing follicle
Antral follicle
Antrum
Primary follicle
Primordial follicle
Primary oocyte
Mature graafian follicle
Responding preovulatory follicle
Secondary oocyte
Polar body
Ovulation
Definitive oocyte
Polar bodies
Sperm penetration

human chorionic gonadotrophin (HCG), Chorulon (Intervet) was therefore administered at a time point corresponding to that of endogenous release of lutenising hormone (LH) and provided the additional stimulus required

4.4.3.1 Influence of Age and Weight on Superovulation.

The sexual maturity of the female has been found to be a major factor in the success of superovulation. The optimum age lies between 3 to 5 weeks, after the first wave of follicle maturation has taken place. This increases the number of follicles capable of responding to the hormones to a maximum.

The nutritional and health status of the mice is also important. Underweight and sick mice with a retarded development will yield reduced numbers of eggs.

4.4.4 Pseudopregnant Mice

Mating a female in natural oestrus with a vasectomised male results in a pseudopregnancy. The mechanical stimulation of the vaginal wall during copulation sends impulses to the hypothalamus which in turn causes the release of prolactin by the pituitary gland. Prolactin induces the corpus luteum remaining following ovulation to produce progesterone. High levels of progesterone inhibit the production of FSH and LH and maintain conditions receptive for the implantation of fertilised embryos in the uterine endometrium. The synthesis of progesterone levels necessary to maintain pregnancy is then taken over by the placenta.

Mice of approximately 6 weeks of age and weighing approximately 20g were used. Heavier mice were avoided as the fat pad surrounding the ovary would be large and would make implantation difficult.

Two females were placed with each sterile male as not all will actually be in oestrus. Females ovulate every four to five days between three and five hours after the onset of the dark period. Males will then copulate with these females at the midpoint of the dark period, this being one to two hours after ovulation. Females were checked for vaginal plugs the following morning.

4.5 Procedures for Preparing Animals for Microinjection

The following procedures were based on those described in Hogan *et al.* (1994). All protocols were carried out in accordance with the guidelines of the Home Office Animals (Scientific Procedures) Act 1986. The genetic background of all animals used was (CBA x C57BL/10) F_1.

4.5.1 Vasectomy

Anaesthesia was achieved by the intraperitoneal injection of 0.3 – 0.35ml of 2.5% Avertin (Aldrich). This gave 20 minutes of anaesthesia as determined by a lack of response to foot pinching.

The abdomen was shaved and wiped with 70% ethanol to sterilise and prevent hair from entering the incision site. Excess ethanol was avoided as its evaporation could cause cooling of the mouse, which is susceptible to hypothermia.

A transverse incision was made through the skin across the abdomen at a level with the top of the leg and repeated in the body wall. If blood vessels were damaged bleeding was stopped by stretching of the body wall with forceps. A stitch in the body wall (4/0 Vicryl with a round bodied needle) was left in position to aid location of the body wall at the end of the procedure.

The fat pad attached to the testis was identified and pulled outside the body cavity. The testis were then laid to one side, the Vas deferens was visible beneath with a blood vessel running along one side.

The connective tissue was pulled away to isolate the Vas deferens and a section was removed by cauterisation. Alternatively two ligatures were applied and the vas deferens severed between the two. This procedure was repeated on the second testis.

As the body wall was lifted the testes were gently pushed back into the body cavity. An absorbable suture, ie Vicryl 4/0 was used to place three horizontal mattress stitches in the body wall. This was repeated in the skin with a silk suture, 5/0 Mersilk with a cutting needle.

The male mouse was then ready for test mating to determine sterility10 days later.

4.5.2 Superovulation

Assuming a 12 hr, 07.00 - 19.00 hr light cycle

DAY 0 : 5 international units (i.u). (50 i.u./ml, made up in phosphate buffered saline, PBS) of Folligon (Intervet) was injected interperitoneally (IP) at 14.00 hr.

DAY 2 : 5 i.u. (50i.u./ml, made up in PBS) of Chorulon (Intervet) was injected IP at 12.00, 46 hrs after injection of the Folligon. The female was placed with a stud male, and mating was assumed to have occurred at 00.00 midnight.

DAY 3 : 09.00 hrs, evidence of mating was sought via the presence of a vaginal plug which was taken to correspond to 0.5 day post-coitus (p.c.).

10 females were superovulated at any one time to ensure that enough embryos were produced for implantation into three or four pseudo-pregnant females.

4.5.3 Recovery of Pre-implantation Embryos

Pre-implantation embryos were recovered from the superovulated females between 11.00 and 12.00 noon on day 3. Later during this day the cumulus cells that form the follicle begin to dissociate and the eggs become increasingly difficult to recover.

Superovulated females were humanely sacrificed by cervical dislocation. The abdomen was sterilised with ethanol and a large transverse incision made through the skin. Either side of the skin was pulled back to reveal the body wall. The body cavity was then opened and the intestines pushed to one side to reveal the uterus. Each of the uterine horns were checked for the presence of seminal fluid from the male.

The ovary and oviduct were revealed by grasping the upper end of the uterus and gently pulling in a caudal direction. They were then separated from the mesometrium. Holding the utero-tubual junction with forceps the oviduct was pulled away from the ovary. Fine scissors were used to isolate the oviduct from the uterus.

The collected oviducts were placed in a 35mm dish of M2 media (Speciality Media, Sigma) at room temperature. Each oviduct was then removed to a microdrop culture (20-40µl individual drops of M2 media overlaid with mineral oil).

Under a stereo dissecting microscope (Nikon) the oviducts were manoeuvred into such a position as to enable access to the enlarged ampulla, the upper region of the oviduct. This was identified by its striated epithelium and the presence of the

embryos within it. Forceps were then used to tear the ampulla and gently coax the embryos out into the media. The oviducts were then discarded (Figure 4.5.3).

Once the embryos from each oviduct were isolated the enzyme Hyaluronidase, (Type IV-S, Sigma) was added to a working concentration of 300μg/ml. This caused the break down of the cumulus cells revealing the embryos. The embryos were not exposed to the enzyme for more than 5-10 minutes to avoid toxic effects. To aid the dissociation of the cumulus cells the embryos were agitated with the handling pipette. Cleaned embryos were subsequently removed to a second flooded dish of M2 in preparation for injection.

Subsequently the embryos were moved through three subsequent 100μl wash drops of M2 to remove traces of the enzyme and discard cumulus cells and debris. The embryos were then kept in M16 media (Cell and Molecular Technologies Inc, Speciality Media) microdrop cultures in a 37°C incubator, 5% CO_2 until ready for microinjection.

4.5.4 Pro-nuclear Microinjection

This procedure was carried out at approximately 14.00 on day 3 which was consistent with two or three hours before pronuclear fusion in the fertilised embryos prior to the first cell cleavage.

Embryos were transferred from the M16 microdrop culture through an M2 wash and into the injection chamber.

The stage was manoeuvred to allow an embryo to be picked up via suction onto the holding pipette. The embryo was then manipulated to an optimum position. This involved placing a pronucleus at the central axis in the hemisphere adjacent to the injection pipette.

Either pronuclei was injected, however the male pronucleus was more often visible as the larger of the two and was positioned closer to the surface of the embryo. In addition, it tended to have only one nucleolus.

Once in place, the suction on the holding pipette was increased to stabilise the embryo during injection. The zona pellucida could be seen to be pulled into the opening of the holding pipette.

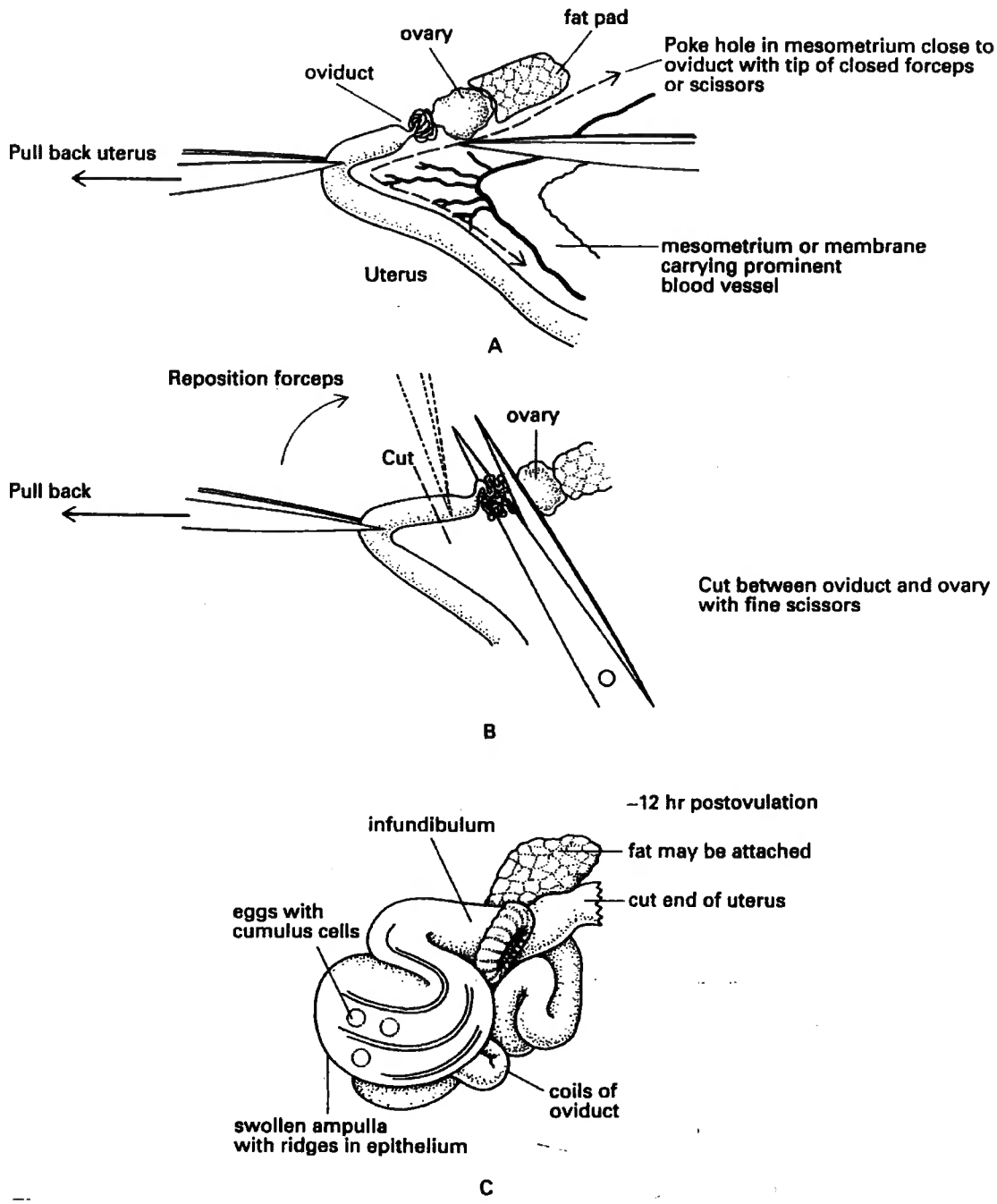


Figure 4.5.3 Recovery of preimplantation embryos
From Hogan *et al.*, 1994. The ovary, oviduct and uterus are separated from the mesometrium (A) and the oviduct is isolated (B). The embryos can be seen in the swollen, striated ampulla. Gentle tearing of this membrane releases the embryos (C).

The point of the injection pipette was brought to above and just beyond the pronucleus and then manoeuvred, through vertical control of the manipulator, into the same plane as the pronucleus. Before insertion, pressure was applied to the air filled syringe driving the injection needle to expel any media that had entered the tip of the pipette. The tip was then drawn back level with the pronucleus and pushed through the zona pellucida and into the pronucleus avoiding contact with the nucleolus. The DNA solution was then injected through the manipulation of the controlling air filled syringe at which point the pronucleus was seen to expand.
Injected embryos were passed through an M16 wash and placed in M16 microdrop cultures in the incubator until ready for implantation.

4.5.4.1 Common problems encountered During Pro-Nuclear Microinjection

I The pronucleus was seen to expand in all directions following successful microinjection. If the pronucleus did not expand a small drop of DNA solution accumulated around the injection site indicating that the pipette had not penetrated the plasma membrane. If the zona pellucida and cytoplasm of the embryo were seen to 'dimple' then the injection pipette was found to have not penetrated the embryo (figure 4.5.4).

II If an injection pipette passed through the nucleoli of the pronucleus on withdrawal the pronucleus was often also removed causing embryo lysis. In this event sticky material from the nucleoli could be seen adhering to the injection pipette and any pipette found to lyse two embryos in a row was discarded.

III Cell lysis occurred within 30–60 minutes and was identified prior to reimplantation. Healthy embryos were seen to have distinct perivitelline space between the egg and the zona pellucida while lysed cells filled the zona pellucida and the cytoplasm was homogenous.

IV Unfertilised embryos identified by homogenous cytoplasm, no evident pronuclei and only one extruded polar body were unsuitable for microinjection. Recently fertilised embryos in which pronuclei were yet to form and older embryos in which the pronuclei had broken down and embryo division was about to commence were discarded. Embryos with three pronuclei were seen and discarded as non-viable, this appears to be a consequence of superovulation. 50 - 80% of embryos were expected to survive microinjection.

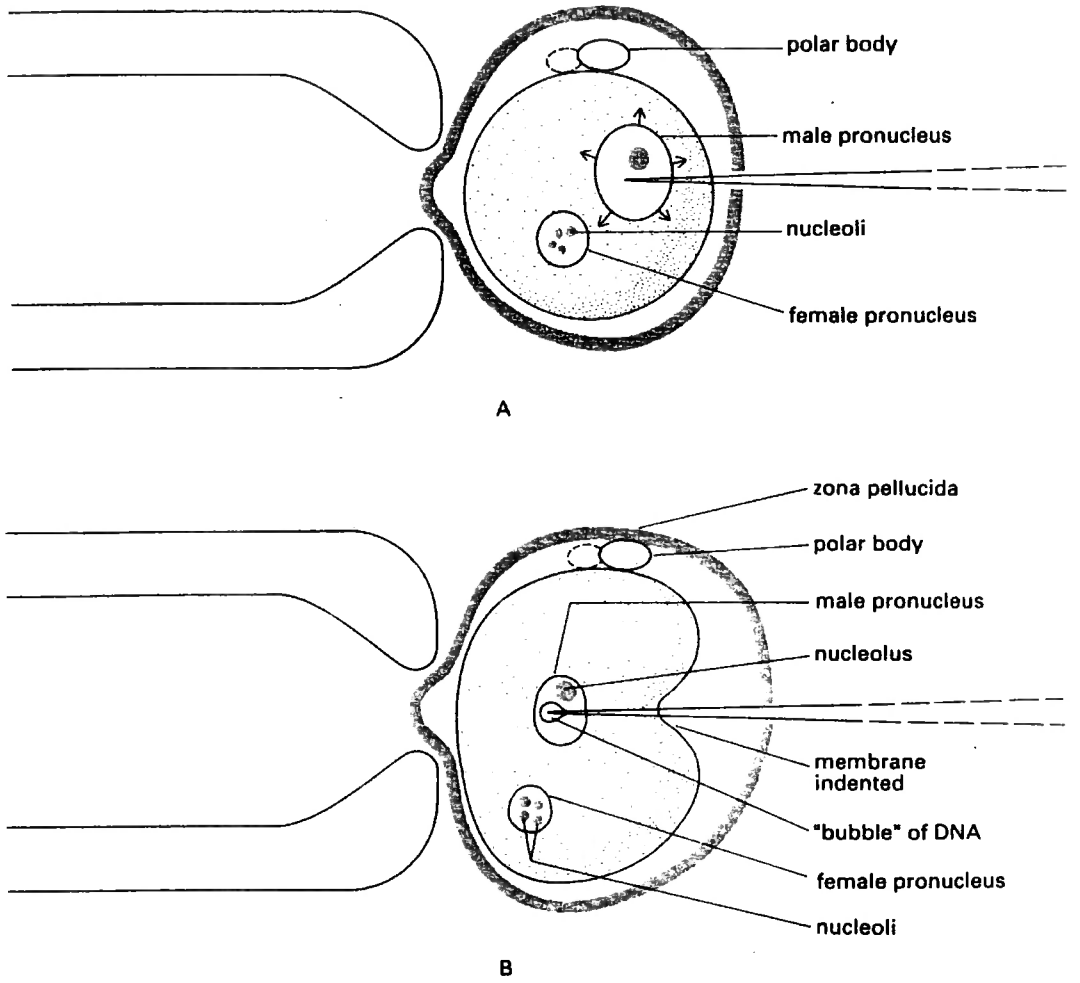


Figure 4.5.4 Microinjection into the pronucleus.
Taken from Hogan *et al.* 1994. Successful injection of the DNA solution into the pronucleus: The injection pipette is seen to penetrate the embryo, and as the DNA solution is expelled expansion of the pronucleus is seen (A). Incomplete penetration of the embryo: If the injection pipette fails to penetrate the embryo the plasma membrane is pushed inwards and as the DNA solution is expelled it forms a visible bubble of solution and the pronucleus does not expand (B).

4.5.5 Oviduct Transfer

Injected embryos were implanted into the oviducts of 0.5 day p.c. pseudo-pregnant recipient females. Approximately 30 embryos were introduced into each mouse, 15 in either oviduct. Of these 30% were expected to survive resulting in litters of around ten pups.

The transfer of embryos into females at an earlier stage of pregnancy than at which they were removed has been found to give the embryos time to advance in development before being exposed to conditions favourable to implantation.

A pseudo-pregnant recipient female mouse was anaesthetised with 0.35ml 2.5% Avertin per 20g mouse and placed on a petri dish lid and manoeuvred onto the dissecting microscope stage.

Embryos were taken through two M2 media washes before being loaded into a transfer pipette filled with M2 media. Two air bubbles were drawn into the pipette followed by 15 embryos immediately adjacent to each other in a minimal volume of media. This was followed by a third bubble and a small volume of M2. The pipette was then placed to one side to ensure the embryos were not disturbed.

A small region of hair from the back of the recipient mouse was shaved between the bottom of the rib cage and the top of the leg. The area was then sterilised with 70% ethanol avoiding excess. A 1cm longitudinal incision was made in the skin and any sub-cutaneous tissue was dissociated to allow free movement of the skin across the back. This was then wiped free of any loose hair with 70% ethanol.

The skin was moved to one side to identify the position of the ovary through the body wall. It could be seen as an orange body with an associated white fat pad. The presence of a nerve was also taken as a strong indication of the correct position.

An incision was made in the body wall, which was stretched to prevent bleeding and a stitch placed within it for location later. The ovary was then pulled out by the associated fat pad. A serrefine clip (Fine Science Tools) was used to hold the fat pad outside the body cavity and pull the attached ovary out to one side.

Under the dissecting microscope the swollen ampulla and the bursa, a highly vascularised membrane covering the oviduct and ovary could clearly be seen (figure 4.5.5).

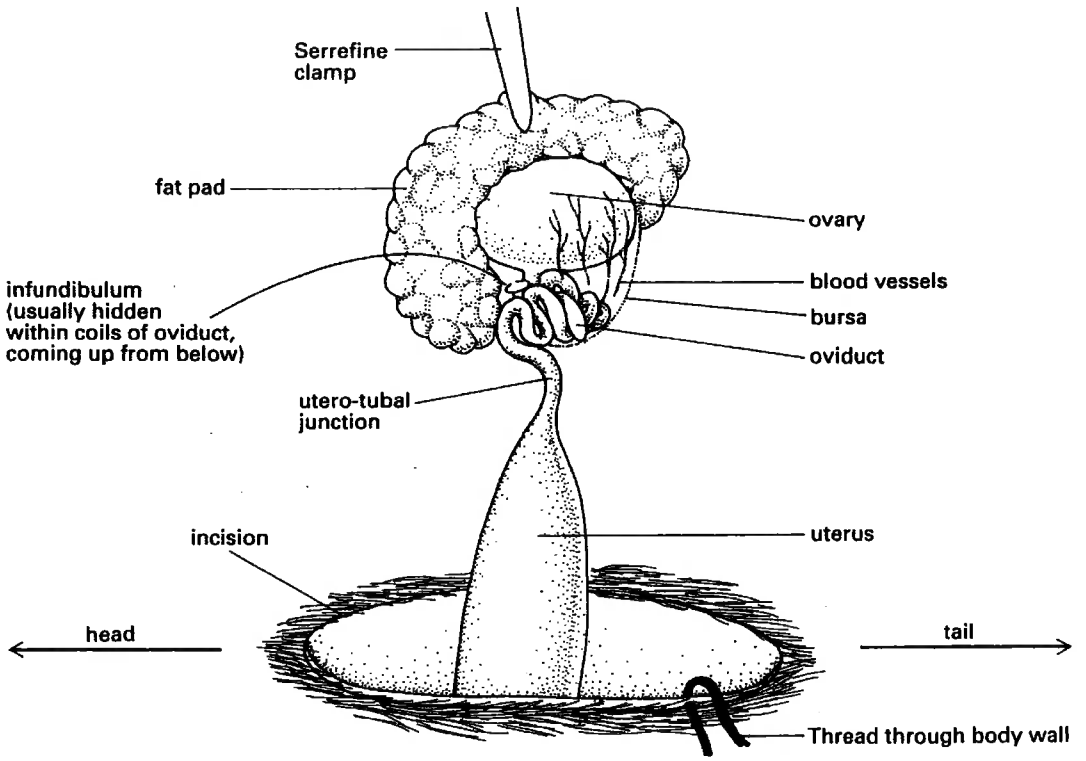


Figure 4.5.5 Transfer of embryos to pseudo-pregnant females
Taken from Hogan *et al.*, 1994. The ovary and oviduct are surrounded by a vascularised membrane called the bursa. Following the application of adrenalin to stop blood flow this membrane can be torn to allow access to the infundibulum. A pipette loaded with embryos was inserted into the infundibulum and the embryos were gently released into the oviduct. The position of the embryos is marked by the position of the air bubbles that were present in the pipette.

Two or three drops of 0.1% adrenaline (Sigma) were applied to the bursa to cause vasoconstriction and prevent bleeding.

A tear in the bursa allowed entry to the infundibulum, the opening of the oviduct, which lies between the ovary and the main body of the oviduct, usually facing the tail.

The loaded transfer pipette was inserted into the infundibulum and the embryos were gently blown into the ampulla. The presence of the air bubbles within the oviduct confirmed correct positioning of the embryos.

The clip was removed, the body wall lifted high and the ovary gently pushed back into the mouse. The skin and body wall were then stretched to the opposite side and the procedure repeated on the second oviduct.

The body wall was closed with a vicryl suture 4/0 and the skin incision was sealed with a wound clip.

A 2-300µl IP injection of PBS following surgery was administered to prevent dehydration and raise blood pressure through an increase in blood volume. The mouse was placed in a box with warm bedding, tissue paper, and left undisturbed under a heat lamp until recovery of the righting reflex and signs of waking.

4.6 Animals Used in the Production of the PMP-22 P1-EGFP Mouse.

During the course of the production of the P1-EGFP transgenic mouse a total of twenty three superovulated females were used. The fertilised embryos recovered from these animals were microinjected with the DNA solution and viable embryos were implanted into pseudopregnant recipients. In all two hundred and forty viable embryos were implanted into eight recipients. Fifty embryos developed to term, and the pups were subsequently screened for the presence of the transgene.

Chapter 5.

Results

Chapter 5: Results

5.1 Identification of Potential Transgenic Founders

Following the successful microinjection and re-implantation of 240 viable embryos the mice were left to progress through pregnancy. Of the total, fifty pups developed to term. The mice showed no developmental or physiological defects and were indistinguishable from wild type mice. Once they had reached the age of three weeks a 2-4mm tail biopsy was taken from each. DNA was extracted using the method described in section 2.3.2. In an attempt to identify potential founders the DNA was screened for the presence of the transgene. Initial dot blot assays gave ambiguous results, mice deemed positive by this method could not be confirmed by the method of PCR in any instance (data not shown). An alternative strategy was adopted, using primers designed against the EGFP sequence, absent from the endogenous genomic DNA, the polymerase chain reaction was used to screen for the presence of the transgene DNA. Three mice designated 4, 11 and 15 were found to have positively incorporated the transgene into their genome. A PCR reaction to screen for the full length construct was then undertaken (Table 5.1.1 and Table 5.1.2, Figure 5.1.3). A reaction product at the expected 1.8kb molecular weight was positively identified by restriction digest (Table 5.1.4, Figure 5.1.3).

Oligo	Sequence 5' to 3'	Position in Transgene
Forward	AGA ACT AGG GCC TCG AAC T	PMP-22 Promoter
Forward	AGC AAG GGC GAG GAG CTG TTC A	EGFP Sequence
Reverse	TTG TAC AGC TCG TCC ATG	EGFP Sequence

Table 5.1.1 Oligonucleotides used to identify potential founder animals

Reaction		Temp (°C)	Time (seconds)
0.8kb EGFP fragment	One cycle: denaturation	92°C	300
	annealing	58°C	45
	extension	72°C	45
	Thirty cycles: denaturation	92°C	45
	annealing	58°C	45
	extension	72°C	45
	Final extension	72°C	300
1.8kb fragment	Two cycles: denaturation	92°C	60
	annealing	68°C	45
	extension	72°C	45
	Fifteen cycles: denaturation	92°	45
	annealing	58°C	45
	extension	72°C	45

Table 5.1.2 PCR protocols used to identify potential founder animals from genomic DNA

The polymerase chain reaction is known to be susceptible to contamination and false positives and so the potential founders were confirmed by Southern blot (section 2.8, Figure 5.1.5). 10μg of genomic DNA from each was digested with the restriction enzyme Bam HI to separate any tandem arrays into individual full lengths of the construct. The DNA was run on an agarose gel and blotted to a nitrocellulose membrane The HindIII/Afl II fragment of P1-EGFP was radiolabelled (section 2.8.2) and used to probe the nitrocellulose filter.

Enzyme	Restriction Site	Fragment Length
Bsp120I	850	850, 725
Eco NI	99	99, 1476
Eco RI	810	810, 765
Eco RV	240	240, 1335
Pst I	68, 424, 815	68, 356, 391, 760

Table 5.1.4. Restriction sites within the P1-EGFP PCR product. Analysis of the fragment generated by the primers PMP-1 and TG-2 used to confirm the identity of the product.

The Southern blot established the incorporation of the P1-EGFP transgene into the genome of the three founder mice. It showed that the number of copies found in each differs. An equal volume of genomic DNA from each mouse was loaded onto the agarose gel. Using densitometric analysis it was shown that founder mouse 15 has incorporated the lowest number of copies of the transgene. The intensity of the hybridised band was 1.2 and 1.8 times greater in founder mouse four and founder mouse 11 respectively.

PCR analysis of Subsequent generations derived from the founder animals has shown that in each line the transgene was successfully transmitted to the F_2 generation, indicating the stability of the transgene within the genome.

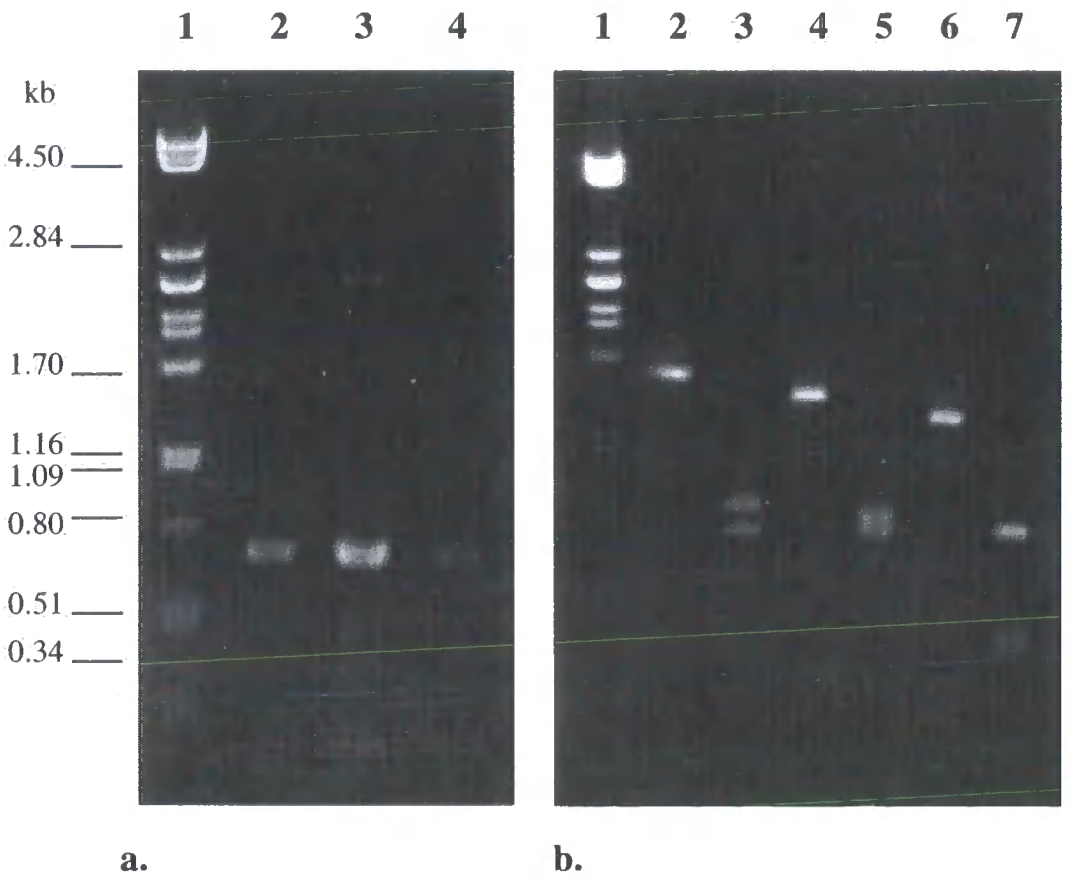
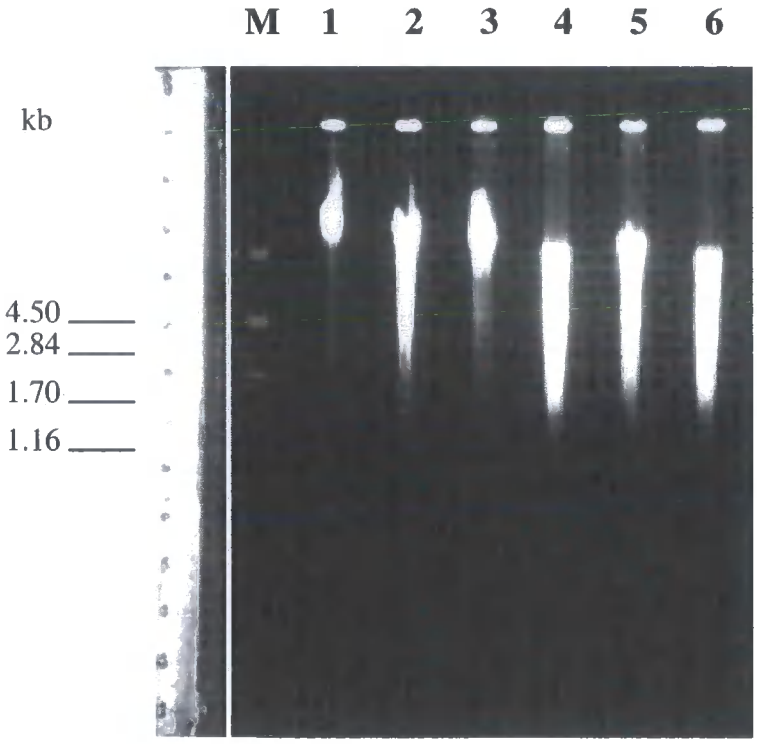
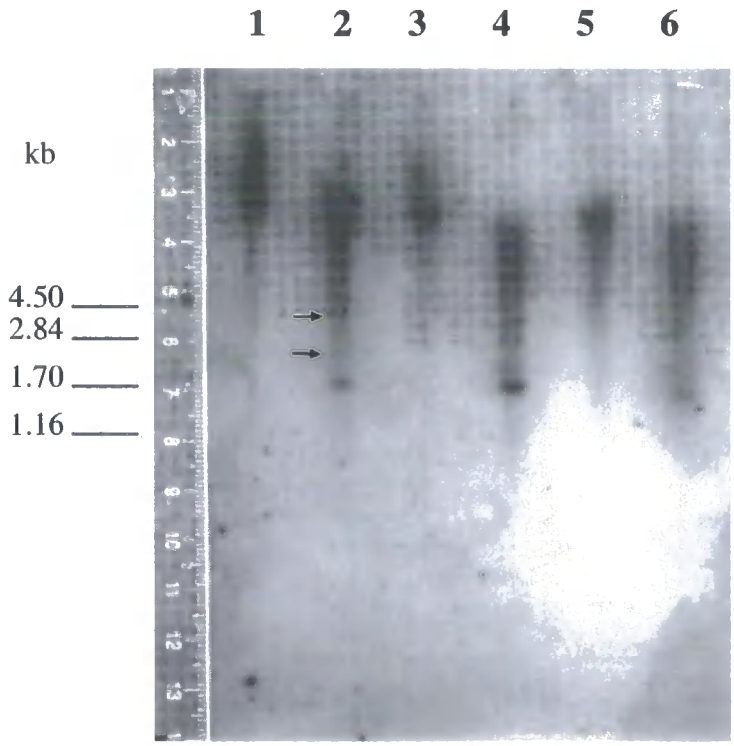


Figure 5.1.3 Identification of Founder Mice by PCR. Three mice were identified as having incorporated the 0.8kb EGFP gene into their genome **a.** Lane 1 molecular weight markers. Lane 2 Male founder four. Lane 3 Female founder 1. Lane 3 Female founder 15. The full length transgene was isolated from the DNA of founder four and identified by restriction digest. **b.** Lane 1 molecular weight markers. Lane 2 1.7 kb PCR product. Lane 3 -7 PCR product digested with Bsp120 I, Dpn I, Eco NI, Eco RI, Eco RV and Pst I respectively.

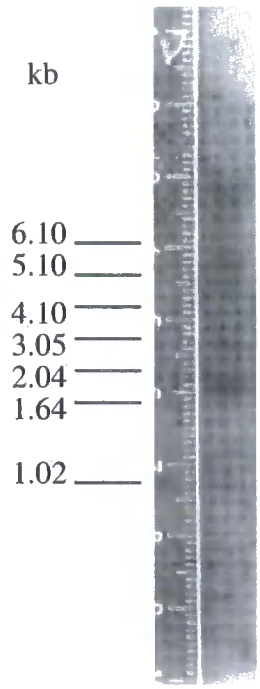
Figure 5.1.5 Positive Identification of Potential Founder Mice by Southern Blot. 10μg of genomic DNA from each potential founder animal was digested with Bam HI and analysed by Southern Blot. Lane 1 and 2 mouse 4 uncut and digested DNA. Lane 3 and 4, and Lanes 5 and 6 mouse 11 and 15 respectively uncut and digested genomic DNA. **a.** The agarose gel was observed against a rule bar to enable identification of the corresponding molecular weights (lane M) on the membrane. **b.** The membrane was probed with the full length of the transgene insert, the Hind III/Afl II fragment of the pP1-EGFP plasmid. Positive bands were seen in each digested lane at the correct molecular weight. In addition two additional bands were faintly observed (arrows). **c.** A subsequent blot of founder 4 shows clearer evidence of the additional bands.


M 1 2 3 4 5 6
kb
4.50
2.84
1.70
1.16

a.


1 2 3 4 5 6
kb
4.50
2.84
1.70
1.16

b.


kb
6.10
5.10
4.10
3.05
2.04
1.64
1.02

c.

5.2 Determining the Expression of the Transgene

Following the identification of potential founder animals their ability to functionally express the EGFP protein (as described by EGFP fluorescence in the targeted cell type) was investigated. The co-culture of Schwann cells and dorsal root ganglion (DRG) neurons based on the method of Wood (1976) was commonly used in our laboratory at this time. Embryonic Schwann cells prepared by this method are able to myelinate and express the myelin proteins associated with more mature Schwann cells under the appropriate culture conditions.

The founder mouse number four was most suitable for initial expression analysis. This male was used for breeding with non-transgenic females that could be sacrificed to study their embryonic progeny. Once mouse number four had reached sexual maturity it was allowed to breed with a wild type female. A vaginal plug was used to determine the time of mating. At embryonic day 15 (E15) the female was sacrificed and the embryos removed from the uterus and decapitated to ensure rapid death. The dorsal root ganglia were removed and cultured as described in section 2.11.2. A great number of litters from founder four and F_1 offspring were studied and in many EGFP positive cells were observed.

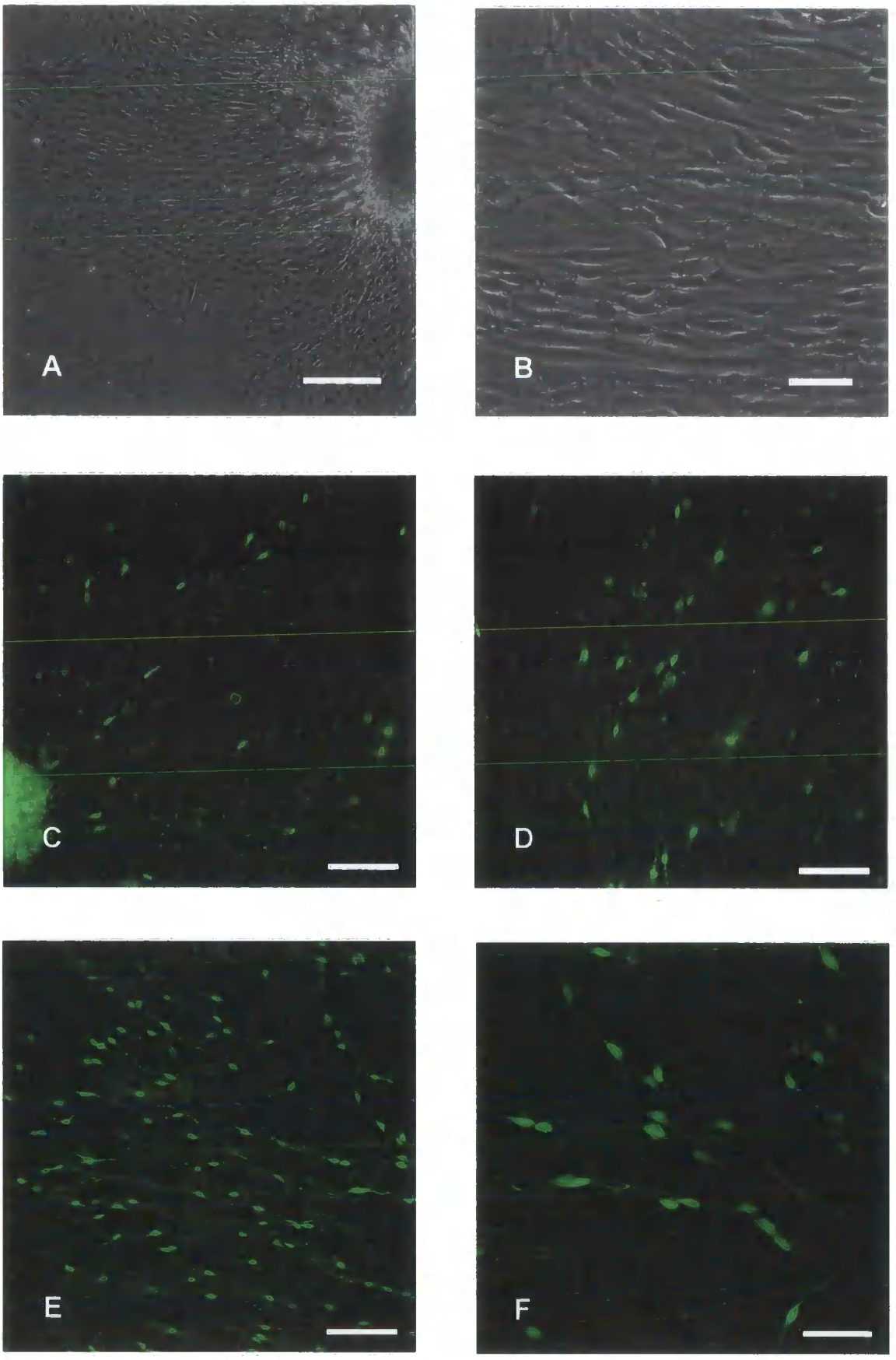
Within twenty-four hours of plating EGFP positive cells could be seen in cultures derived from three out of the ten embryos. Initially the fluorescent cells were predominantly within the ganglion and the immediate area surrounding it. Forty-eight hours after plating the positive cells had become more elongated and could be seen at greater distances from the ganglion (Figure 5.2.). Once the DRG axons had spread within the culture dish the fluorescent cells could be seen throughout. The positive cells were localised to specific regions of the dish, running in tracts throughout the culture (fig 5.2. c and d).

In cultures derived from individual pups of the same litter, there was variation in the proportion of cells within each culture that were fluorescent. Expression ranged from those in which only one or two cells expressed EGFP within each plated ganglion to those in which approximately 30% of cells were fluorescent.

The DRG co-culture system contains Schwann cells, axons and fibroblasts. Of these, both axons and Schwann cells display polarity and long, slender processes. However the two can still be distinguished by morphological criteria. The cell bodies of the DRG neurons are located within the ganglion and their processes extend out from it. Schwann cells however are bipolar with distinct cell bodies and the EGFP positive cells found throughout the culture had these features. The processes of adjacent EGFP positive cells also generally ran in parallel, a morphology that more closely resembles that of Schwann cells than neurons. A small number of positive cells towards the periphery of the culture were more flattened with more numerous processes of shorter length.

Studies showed that fluorescent cells were present within the ganglia as they were removed from the embryo, and that therefore the EGFP expression is not a result of the culture conditions or isolation of the cells from *in vivo* regulatory signals.

Figure 5.2 Transgenic Mice were Investigated to Determine their Ability to Express the Transgene. Dorsal root ganglia co-cultures of Schwann cells and neurons from E15 embryos of transgenic mice. *In vitro* the cultures were identical to that of wild type mice. **A** and **B**. The Schwann cells radiate out from the central ganglion. Scale bar 200μm and 50μm respectively. **C-F**. Under excitatory light distinct cell bodies and elongated processes can be seen to express EGFP. (A and B phase contrast microscopy, C and D fluorescent confocal microscopy). Scale bar 100μm C-E and 50μm F.


A
B
C
D
E
F

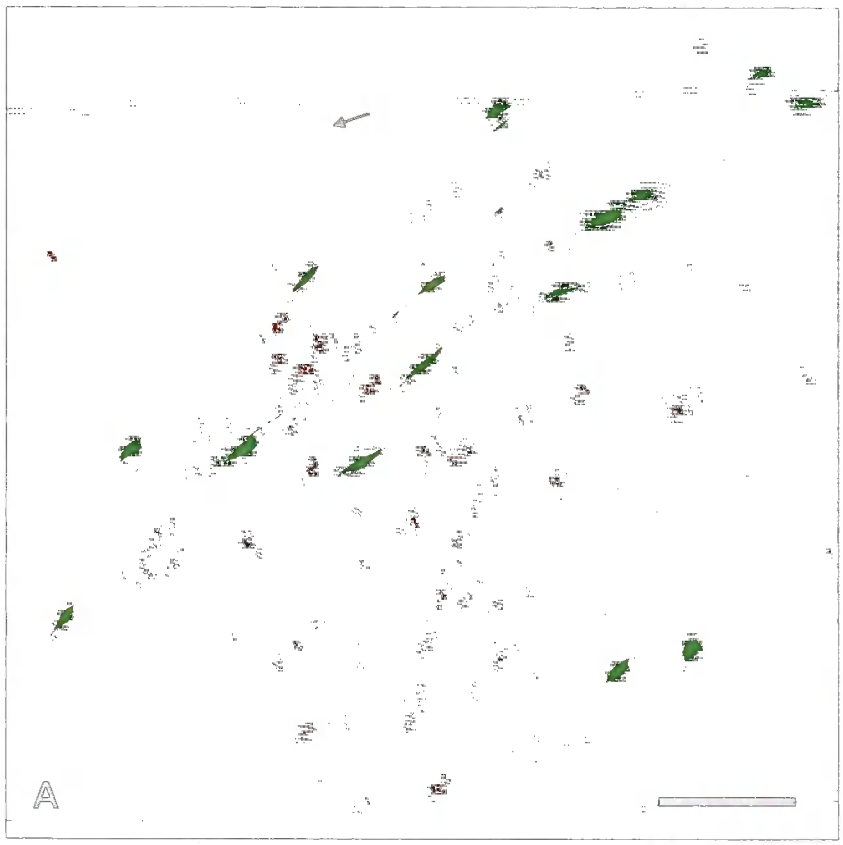
5.2.1 The Site of Transgene Expression

To confirm the identity of the cell type in which the transgene was being expressed positive co-cultures were incubated with antibodies against both Schwann cells and neurons. Co-cultures were fixed in 4% paraformaldehyde and incubated with the S-100 monoclonal antibody (Sigma) or either of the neurofilament NF-68 or NF-200 antibodies (Sigma, section 2.11.2) and labelled with fluorescent secondary antibodies.

The results showed that the EGFP fluorescence co-localised with the S-100 antibody (Figure 5.2.1.1). The full span of an individual Schwann cell labelled with S-100 could be difficult to detect, however the EGFP positive cell bodies were clearly identified with the S-100 antibody and some double labelled processes were also seen.

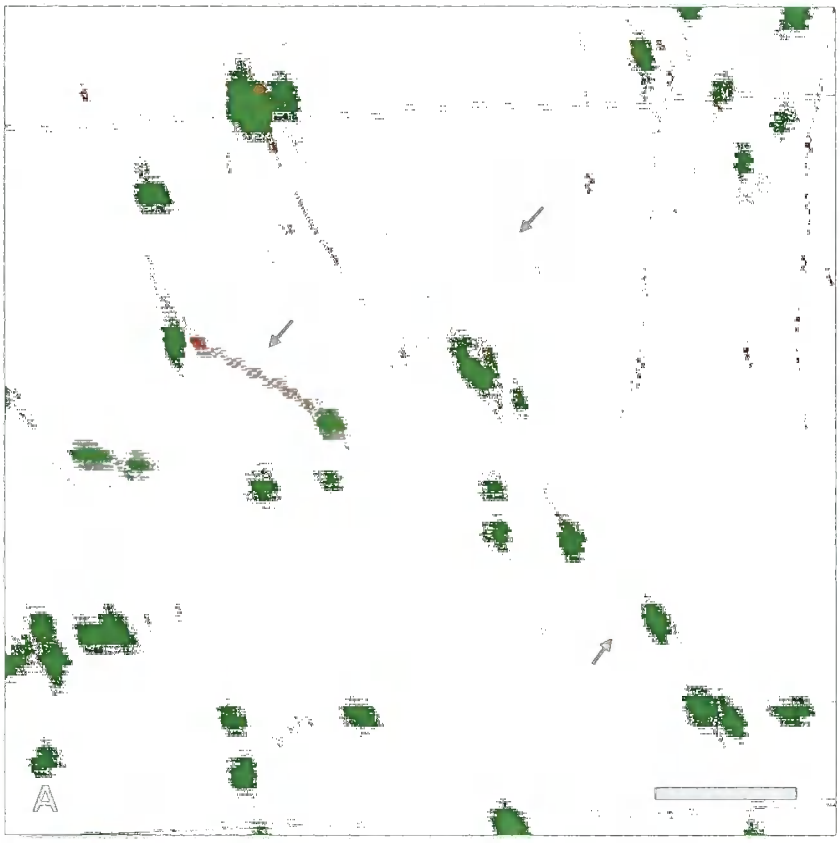
The expression pattern of EGFP and the neurofilament (NF) antibodies were shown to be distinct (Figure 5.2.1.2). NF positive fibres were seen to originate at the ganglion and spread across the culture dish. No NF positive cell bodies were found away from the ganglion. At higher magnification the fluorescent processes of the transgene expressing cells can be seen to intertwine around those of the neurons (Figure 5.2.1.2). In many cases the fluorescent cells appeared in very close apposition to the neuron.

Figure 5.2.1.1 Immunostudies Identified the Positive Cells as Schwann Cells. Immunostaining of the DRG co-cultures with the S-100 antibody (Alexa-fluor 546 secondary antibody, Molecular Probes) specific to Schwann cells in the PNS corroborated morphological identification of the cells. **A**. EGFP fluoresecent cell bodies can be seen to run in a swathe among other S-100 positive Schwann cells, bordered on one side by a group of fibroblasts (arrow). **B**. Only a proportion of the Schwann cells present is seen to fluoresce. Distinct localisation of the EGFP positive Schwann cells is seen. Scale bar 100μm.


A


B

Figure 5.2.1.2 The Relationship between Transgenic Schwann Cells and Neurons was Examined. Anti-neurofilament antibody NF-68 (TRITC secondary antibody, Kirkegaard and Perry Inc.) was used to label neurons within the transgenic DRG co-culture. Fluorescent confocal microscopy was able to show the fine interaction of the two cell types. A. The Schwann cell processes are seen to extend around the neuronal fibre as indicated by the arrows. Scale bar 40μm. B. At times the Schwann cell is seen along the nerve fibre, at others it circumnavigates the processes. Scale bar 20μm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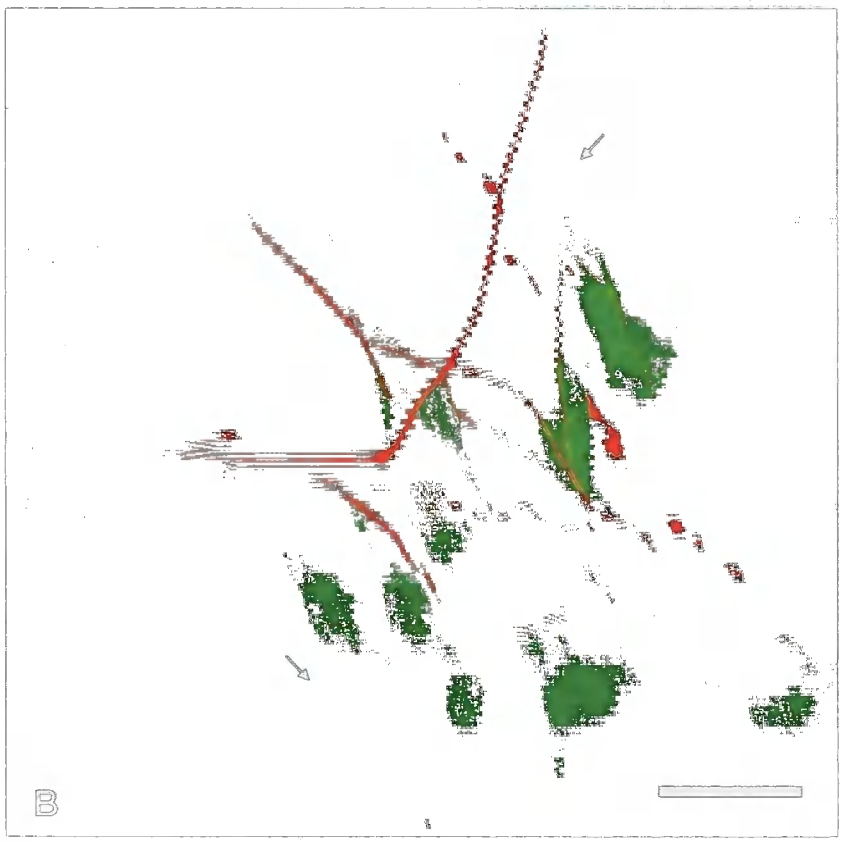
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5.2.2 Non-neural Expression of the Transgene

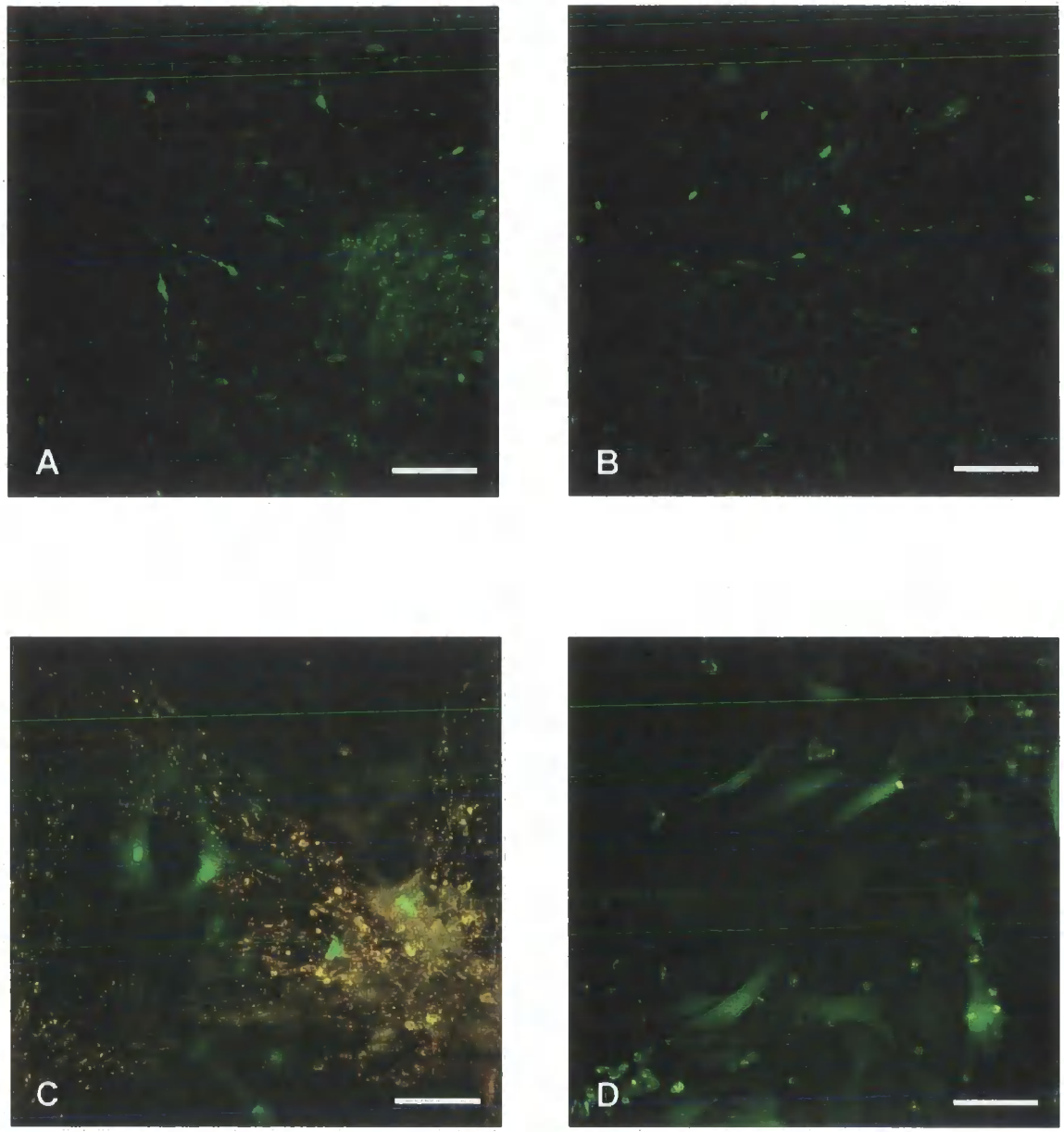
In order to assess the expression pattern of the transgene outside of the nervous system a number of non-neural sites were investigated in at least three different transgenic embryos. Briefly sections of tissue were taken from an E15 embryo that was seen to express EGFP fluorescence. They were dissociated and plated into 35mm tissue culture dishes (section 2.11.3). Within twenty-four hours some cells had attached to the substratum and continued to divide.

In the heart culture large sections of tissue that had adhered to the surface continued to beat independently for some time. However few cells migrated out of these regions and the cultures subsequently died. No evidence for EGFP expression was seen at this stage.

The culture of cells from the lung, liver and skeletal muscle were maintained between one and three weeks before being fixed and mounted. In the culture derived from skeletal muscle of the hind limb a number of distinct cell types were observed. These included myotubes and bright cells with bipolar morphology. In addition unidentified cells with less defined morphologies that resembled fibroblasts were present. Six days after plating, once the cultures had become confluent they were assessed for the expression of EGFP using a Zeiss Axiovert inverted fluorescence microscope. A small number of cells showed weak EGFP fluorescence (Figure 5.2.2A and B). Morphologically these cells resembled Schwann cells seen *in vitro*, with clearly defined cell bodies and fine, elongated processes. Positive cells were found in isolated areas and were not diffuse throughout the muscle. Myotubes were not found to be positive for EGFP expression.

In both the lung and liver cultures a number of different cell types could also be described on the basis of their morphology. Flattened cells similar to the fibroblast morphology were abundant. In addition in each were regions that appeared more organotypic in nature. The cells were together in clusters from which other cells migrated out. In these organotypic regions in both the lung and liver cultures a number of very faint EGFP fluorescing cells were seen (Figure 5.2.2C and D). Unlike the skeletal muscle culture these cells did not display a Schwann cell like morphology. The

Figure 5.2.2 Expression of the Transgene was Found in Non-neural Tissues. Previous reports of the contribution of the P1 promoter and exon 1a transcript in non-neural expression of PMP-22 have been confirmed. **A and B.** EGFP fluorescence was seen in cultures from the skeletal muscle. The morphology of the positive cells is reminiscent to that of Schwann cells. **C and D.** Fluorescent cells with a less defined morphology were found in cultures derived from the lung and liver respectively. Scale bar 100μm.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positive cells were large and flat with much cytoplasmic material. In the lung culture the cell nuclei were strongly fluorescent (Figure 5.2.2C).

The Zeiss microscope possesses a mercury lamp as the source of illumination, white light is passed through specific filter blocks to provide light of the correct wavelength required to excite the fluorescent species of interest. In this way the intensity of the illumination and the sensitivity of the observation of the fluorescence cannot be enhanced. The level of expression of EGFP, the intensity of the signal was greater in Schwann cells from DRG co-cultures than the potential Schwann cells in the skeletal muscle culture. In turn the expression seen in the unidentified cell types of the lung and liver was substantially weaker again.

5.2.3 Summary

Three transgenic lines have been established with varied copy number of the transgene. Expression of the construct has been demonstrated in animals derived from founder four. In the remaining lines eleven and fifteen the transgene was shown to be transmitted to subsequent generations, but expression has not been seen in cells within the dorsal root ganglia cultures. Extensive study of founder line four has been carried out and a concerted programme of investigation into the remaining two lines should be undertaken to ascertain their ability to express the reporter construct.

Within the dorsal root ganglia culture system, Schwann cells, axons and fibroblasts interact. The morphology of the positive cells was indicative of the expression of the transgene in its target cell type. That expression was restricted to Schwann cells within this system was confirmed using a monoclonal antibody against the S-100 protein that is specific to Schwann cells in the peripheral nervous system. Staining with the anti-neurofilament protein NF-68 confirmed that neuronal cells did not express the transgene. In addition the expression of EGFP driven by the P1 promoter has been described in a number of non-neural locations. EGFP expression in the skeletal muscle may in fact be derived from innervation of the muscle by the sciatic nerve. However in the lung and liver, whilst innervation cannot be ruled out at this stage the morphology of the cells expressing the EGFP is not consistent with that of Schwann cells.

5.3 Investigation of the Regulation of Transgene Expression *In Vivo.*

5.3.1 Observation of Neonatal peripheral Nerves

It was noted that *in vitro* within the outgrowth of each ganglion only a proportion of the Schwann cell population shows EGFP expression (section 5.2.1). It is possible that this is due to the developmental age of the cells as they are removed from the embryo. Once in culture the necessary signals required to initiate expression of the transgene may be absent. Since the transcription of PMP-22 through the P1 promoter increases from the day of birth through the early postnatal period, analysis of Schwann cells from older animals could be expected to show enhanced EGFP fluorescence as a result of this developmental increase.

The sciatic nerve is a major myelinated peripheral nerve of the hind limb. It is easily accessible and is clearly visible beneath the translucent muscles of neonatal mice. In order to follow the expression of the transgene during postnatal development the sciatic nerve was dissected from transgenic mice between the ages of five to forty days, (P5 to P40).

A section from the tip of the tail of each newborn mouse was taken and the genomic DNA extracted from it (section 2.3.2). The DNA was then screened for the presence of the EGFP gene by PCR (section 5.1). Positive mice at different ages were taken and their sciatic nerves removed (section 2.11.4).

When examined by phase contrast light microscopy the myelin sheath could be seen in many of the dissected nerves. A number of nodes of Ranvier were also evident as opposing myelin internodes abutted. The nerve fibers were then assessed for EGFP expression by fluorescent confocal microscopy.

During the course of this experiment sciatic nerves were taken from animals of each line and across an age range that incorporated the timescale of upregulation of the PMP-22 mRNA and protein.

Mice from line four, which is known to express the transgene *in vitro*, were studied at P10, P15 and P21. During this time levels of PMP-22 expression continue to rise from

birth, with the peak of myelination occurring at approximately P21. No EGFP expression was seen in any of these animals. A comprehensive study of line eleven included investigation of nerves from animals from P14 the time of peak PMP-22 expression to P40 and intermediate points in between. This follows the increase in myelin and myelin protein expression through its peak and beyond. Despite this at no time was EGFP fluorescence observed in any of these nerve fibres. Neonatal mice from line fifteen were studied at P5 and P10. In addition the sciatic nerves of an adolescent (ten weeks old) and an adult transgenic mouse were observed. Again no fluorescence was found in any instance. A summary of the mice used throughout this section of the study is found in Table 5.3.1.1.

To confirm that the EGFP expression seen previously was not an artifact of the *in vitro* conditions in which the DRG co-cultures were maintained, Schwann cells were prepared from the sciatic nerve of P5 neonatal mice. The sciatic nerves were isolated as described (section 2.11.4) and a section from each mounted for immediate appraisal of EGFP expression. The remaining nerves were subsequently minced and treated enzymatically to dissociate the cells as described in section 2.11.1. This culture contains Schwann cells and fibroblasts. EGFP fluorescence was absent from the sciatic nerves at the time of removal from the animals, and subsequently following time in culture. This further indicates that the *in vitro* conditions are not determining the expression of the transgene (Table 5.3.1.2).

5.3.2 Discrepancies in Expression within DRG Co-cultures

Concomitant to this series of experiments consecutive dorsal root ganglion dissections derived from animals that had previously given rise to transgenic embryos, had failed to produce cultures that showed any EGFP expression. These additional discrepancies between the observed behaviour of the transgenic line, and the expected behaviour, suggested that these embryonic mice might also posses the transgene but fail to express it. To address this issue, during subsequent dissections, following plating of the DRG a section of tissue was taken from each embryo. The genomic DNA was extracted from this and analysed by PCR for the presence of the EGFP gene (section 5.1).

Founder Line	Age	Number of Animals Studied	EGFP Expression
4	P5	6	X
	P10	2	X
	P15	2	X
	P21	3	X
11	P14	1	X
	P21	4	X
	P25	2	X
	P29	1	X
	P35	2	X
	P40	1	X
	4 weeks	1	X
15	P5	1	X
	P10	1	X
	P15	3	X
	P21	2	X
	10 weeks	1	X
	adult	1	X

Table 5.3.1.1 Neonatal animals investigated for expression of the transgene. Details of the number of neonatal animals and their ages from which sciatic nerves were isolated and analysed for expression of EGFP. In no instance was EGFP fluorescence observed.

Date of Dissection	Founder Line	Number of Pups	Expression on Removal	Expression After Culture
19.08.99	4	2	X	X
08.09.99		4	X	X

Table 5.3.1.2 Expression of the transgene in Schwann cells isolated from sciatic nerve. Sciatic nerves from transgenic mice of two litters were removed from p5 neonates. Initial observation failed to find evidence of EGFP fluorescence. Schwann cells were prepared from the sciatic nerves and grown in culture. No subsequent expression of EGFP was seen.

A comparison could then be made between the fluorescent status of the Schwann cells from each mouse in culture and the genomic nature of each individual.

Of the sixteen litters across all lines studied in this way fourteen contained mice that, although positive for the incorporation of the transgene failed to express EGFP in their Schwann cells, both as they were removed from the embryo and during subsequent time *in vitro*. A summary of each litter and the expression patterns of each embryos are given in Table 5.3.2.

To insure against contamination of PCR reactions the results from three of these litters were confirmed. Duplicate dishes containing ganglia from each embryo were maintained and allowed to proliferate. Genomic DNA was purified from the monolayer of cells by the method described in section 2.3.2.1 and screened by PCR. Each sample repeated in this way corroborated the previous PCR result.

5.3.2.1 Southern Blot Analysis of Litters

Southern blot analysis of two litters from which mice both express the transgene and were non-expressing carriers was undertaken to determine if there was any notable difference between the animals in the way in which the transgene was transmitted and to further substantiate PCR results.

The male mouse 4.3, an F_1 offspring of founder four had previously been shown to give rise to EGFP expressing embryos. A litter founded by this male was sacrificed at E15 and the dorsal root ganglia cultured from the recovered embryos. Immediately following dissection one embryo designated 4.3/2 showed strong EGFP fluorescence. DRG from each embryo were cultured and subsequent examination showed very limited expression of the transgene, one or two fluorescent cells per ganglia in cultures from embryos 4.3/4 and 4.3/7.

Genomic DNA was extracted from tissue of all eight pups. Subsequent analysis by PCR identified five of these, including 4.3/2, 4.3/4 and 4.3/7 as positive for the transgene. In addition mice 4.3/1 and 4.3/5 also contained the EGFP gene. Primers spanning the full transgene sequence were utilised in a second PCR reaction to ensure that the full length had been incorporated (Figure 5.3.2.1).

Date of Dissection	Founder Line	Number of Pups per Litter	PCR+ve/EGFP-ve	PCR+ve/EGFP+ve
31.08.99	4	4	4	0
18.09.99	4	7	6	2
03.10.99	4	3	0	0
11.10.99	4	8	5	3
13.10.99	11	8	7	0
03.11.99	11	11	7	0
23.11.99	15	12	6	0
06.12.99	4	8	4	1
09.12.99	4	2	0	0
18.12.99	11	11	11	0
20.12.99	11	8	7	0
14.01.00	4	5	4	1
19.01.00	4	11	8	3
20.01.00	4	6	3	0
18.02.00	4	8	6	0
01.06.00	4	10	3	0

Table 5.3.2 Details of the number and nature of E15 embryos studied. This table demonstrates the number of E15 DRG dissections that have given rise to mice that are positive by PCR but do not express the transgene and those that do express the EGFP protein.

A total of 10μg of genomic DNA from each pup was digested with excess amounts of the restriction enzyme Bam HI and the subsequent agarose gel was blotted to a membrane and probed for the presence of the transgene (section 5.1).

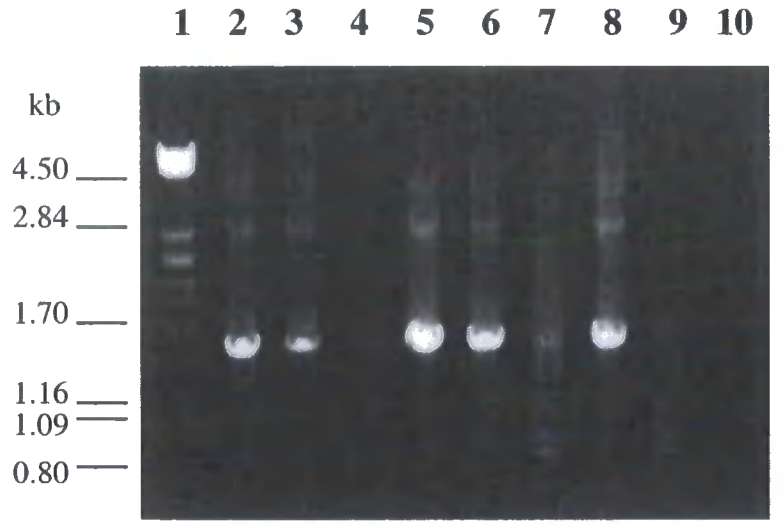
The Southern blot confirmed that mice 4.3/1, 4.3/2, 4.3/4, 4.3/5 and 4.3/7 were positive for the incorporation of the transgene into their genomic DNA. In addition the restriction pattern of each is almost identical. Approximately equal amounts of DNA were loaded and transferred to the membrane. Without internal control DNA probes a specific copy number for each cannot be determined accurately. No obvious discrepancy was seen between either the PCR positive/EGFP negative mice and those that were positive for both or between the mouse that expressed strong EGFP expression and those that did not.

This data has implications for the study of the transgene in neonatal animals, it means that at no time can it be certified that an absence of expression of EGFP at any time point during development is due to the regulation of the transgene. In each instance it is possible that the animal is not able to express the transgene. A mechanism for determining individuals that are able to express the transgene is needed before the analysis of the temporal pattern of EGFP expression can be achieved successfully.

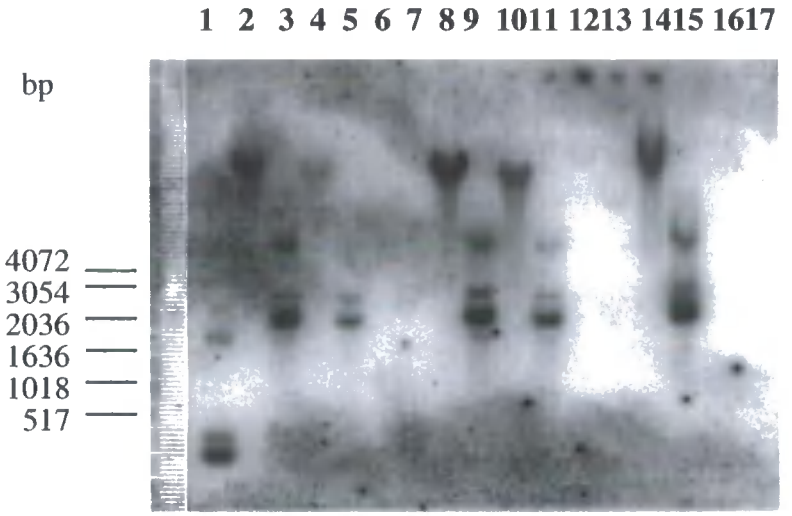
5.4 Investigation into the Transgene Expression Observed *In Vitro*

The focus of this work was then returned to the study of the transgenic Schwann cells *in vitro*. Two key observations were explored.

Variation in the extent to which Schwann cells from different embryos of the same litter express the transgene were observed, and the relative level of expression between two embryos could be determined from the ganglia as they were removed from the embryo. It was possible that this reflected a genetic characteristic of each embryo, but it could not be ruled out that individual embryos have developed at different rates. *In vivo* signals that upregulate the transcription of the construct in more advanced embryos may have been absent from this culture system preventing further stimulation of expression in less



a.



b.

Figure 5.3.2.1 Confirmation of the presence of the transgene fragment within the genome of mice that do not express EGFP. The initial PCR reaction was duplicated using primers against the full P1-EGFP sequence **a.** Lane 1 molecular weight markers. Lanes 2-9 DNA from embryos 1-8 (disection 11.10.99) respectively. Lane 10 No DNA control reaction. This was further corroborated by Southern blot analysis of the mice. **b.** Lane 1 molecular weight markers. Lane 2 and 3 DNA from embryo 1 uncut and digested with Bam HI. Lanes 4-17 DNA from embryos 2 to 8 uncut and Bam HI digested respectively.

developed embryos in culture. If this were so, the effect of this signal is long lasting as the expression of EGFP in culture is not seen to decline with time.

Schwann cells in DRG cultures radiate out from the ganglion and are orientated longitudinally along these lines. Observation showed that the positive cells within a culture were localised to defined areas. A number of fluorescent cells could be seen to lie within a swathe of cells (Figure 5.2.1). Occasionally these regions were bordered by fibroblasts (refer to Figure 5.2.1A), others had no evident structural barrier that could account for this compartmentalisation of expression.

In an attempt to determine the nature of factors that could influence both the expression of the EGFP protein and the spatial pattern of expression two components of the culture system that are intrinsically involved in the regulation of gene expression and myelination were considered.

Axonal contact has been shown many times to influence Schwann cell behaviour and it is intimately involved in the regulation of the myelin proteins. PMP-22 is initially expressed in the cytoplasm of Schwann cells. Following axonal contact and the deposition of a basal lamina it is translocated to the plasma membrane (Pareek *et al.*, 1997; Notterpek *et al.*, 1999). If axonal contact is lost expression decreases and recovers when regeneration is complete (Spreyer *et al.*, 1991; Welcher *et al.*, 1991). In addition to the requirement of neuronal contact the formation of an organised basal lamina is intrinsic to the myelination system and has been reported to influence myelin protein regulation (Fernandez-Valle *et al.*, 1993).

Subsequent investigation aimed to determine the influence of these factors on the Schwann cells and transgene expression.

5.4.1 Axonal Contact

To determine if the axonal presence in the DRG cultures was sufficient to cause EGFP expression the ganglia from each culture dish were excised. A glass pipette was pulled in a bunsen burner flame to a fine, clean point. This was then used to carefully cut around the ganglia severing the axonal processes from the neuronal cell bodies. The ganglionic masses were then removed. Parallel control co-cultures were immunostained with anti-NF antibodies (section 2.12.2) at various time points following excision. At the earliest

time studied, 48 hours after ganglion removal, the axonal processes had degenerated and no NF staining was seen (Figure 5.4.1 and F). Occasional single neurons were found, and these were associated with individual neuronal cell bodies that were separate from the main ganglionic mass and persisted in the culture.

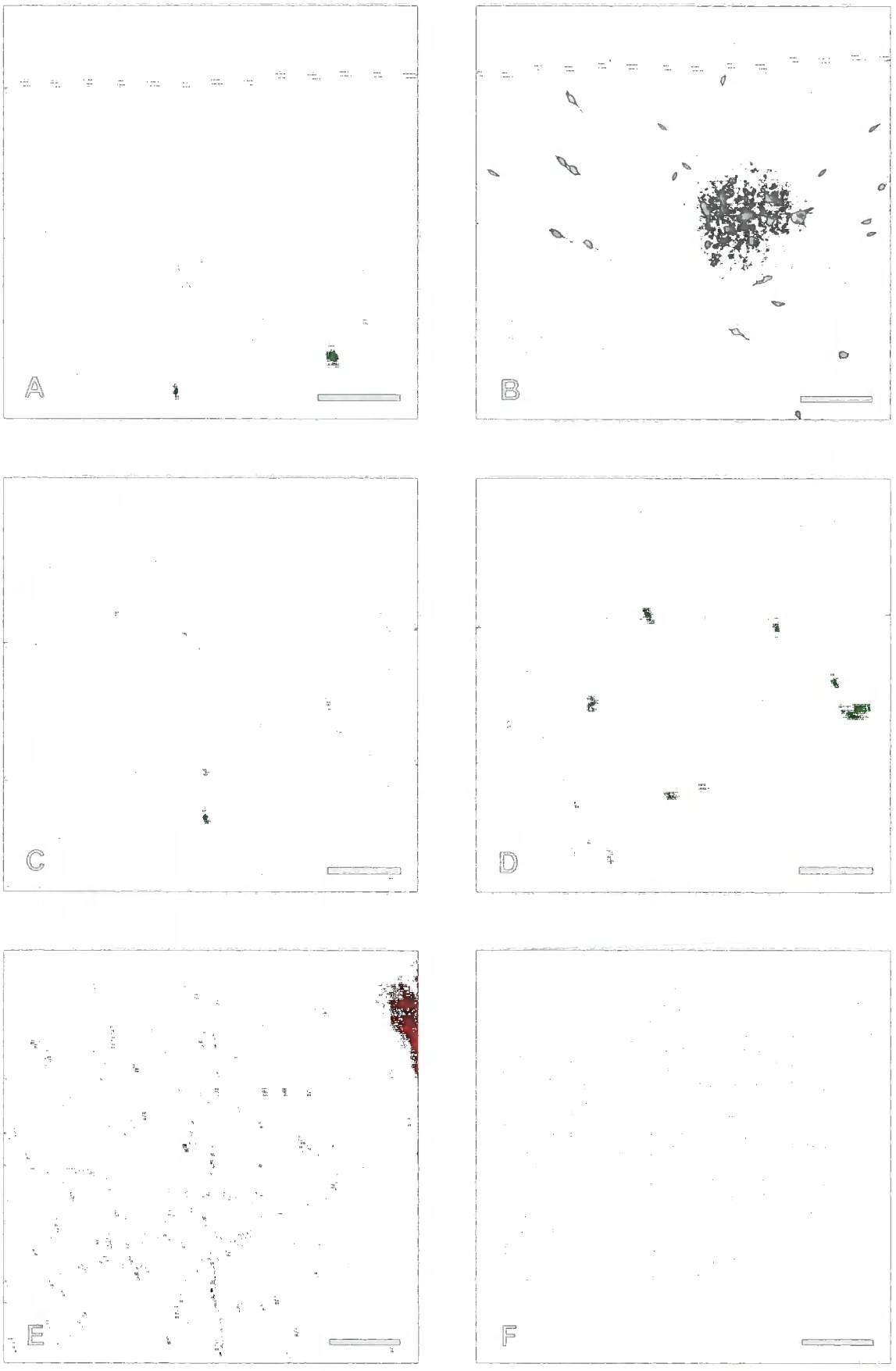
Following axonal removal the cultures were followed for up to three weeks throughout which time EGFP fluorescence remained. Any change in the intensity or proportion of expression was not significant enough to be readily noted. In addition the morphology of the Schwann cells altered following axonal removal. The dense nature of the network of Schwann cells surrounding each former ganglion became more diffuse. The Schwann cells lost some of the bipolar nature of their morphology and processes became shorter and more numerous (Figure 5.4.1). The restricted distribution of the positive cells remained in part, although due to the altered morphology and the reduced density of the Schwann cells the periphery of such regions became less well defined.

5.4.2 Basal Lamina

Schwann cells are capable of secreting components of the basal lamina prior to the initiation of myelination. However only in the presence of neuronal contact and the secretion of collagen type IV, as afforded by the influence of serum and ascorbic acid in the culture medium, is this matrix organised into a full basal lamina. The tissue culture regime used here maintained the co-cultures in a chemically defined media that was not supportive for the formation of a basal lamina. With this in mind the use of the P1 promoter to drive PMP-22 expression seen here, deduced through the expression of the EGFP reporter gene, is independent of a fully formed basal lamina.

Typically DRG co-cultures are plated onto coverslips coated with collagen type I and poly-lysine. This provides a substratum to which the ganglia can adhere and axonal and Schwann cell outgrowth can occur. To investigate if either of these components were preventing or stimulating the expression of EGFP, or indeed if other proteins associated with extracellular matrices could influence the level or restriction of transgene expression, ganglia were plated on a range of different substrates.

Figure 5.4.1 The Removal of Axons From the DRG Co-cultures Affects the Morphology of the Remaining Schwann Cells. A and B. Schwann cells in DRG co-cultures radiate out from the ganglion and are seen to run parallel to one another. Observation has shown that the pattern of fluorescent Schwann cells within such a system is restricted to localised areas. Scale bars 200μm and 100μm respectively. **C and D.** Following removal of the neurons from the culture the Schwann cell morphology becomes less well defined. Bipolarity becomes less obvious and cells no longer orientate in the same direction. **E and F.** DRG co-cultures immunostained with the NF-68 antibody and the TRITC fluorescent secondary antibody. The extent of the network of neurons can be seen in Fig E. The ganglia have been removed from the culture in Fig F, in this control experiment the excision of the DRG ganglia from the culture results in the degeneration of the neurons within 48 hours. Scale bar 100 μm.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Dorsal root ganglia were removed from two positively expressing embryos from different litters as described in section 2.11.2 and plated on collagen/poly-lysine coated coverslips. After 3-4 days in culture the extent of fluorescence was assessed and images taken for reference. Ganglia were then excised from the dish using a glass pipette pulled to a fine point in a bunsen flame and re-plated onto one of a range of substrates (section 2.9.6).

These experiments were designed to investigate the effect of different substrates on the pattern of expression of the transgene in cultures that were already expressing EGFP prior to the re-plating procedure. In an attempt to "turn on" the transgene in embryos positive by PCR but negative for the expression of the transgene, ganglia from such an embryo were also plated on alternative substrates to observe any induction in expression.

On all substrates the Schwann cells were seen to retain EGFP expression (Table 5.4.2.1). A limitation of this series of experiments was the reduced ability of the DRG neurons to extend efficiently on a number of the extracellular components. This prevented axonal stimulation of Schwann cell proliferation. Therefore although the presence of EGFP fluorescence could be seen in Schwann cells within the transplanted ganglia, limited migration of cells into the culture dish meant that assessment of any change in the distribution of expression was restricted on the collagen only, poly-lysine only and laminin coated coverslips (Figure 5.4.2.2). Ganglia plated on coverslips that had no coating were also able to maintain EGFP fluorescence within the area of outgrowth.

The Matrigel substrate proved permissive for the adherence and outgrowth of DRG neurons. Schwann cell proliferation was strong and the cells migrated throughout the radius of the axons. However the morphology of the Schwann cells was altered from that observed in the collagen/poly-lysine controls. Previously individual Schwann cells were bipolar with fine extended processes. The cell body was small and central and appeared three dimensional with phase contrast optics. Following plating on the Matrigel substrate many of the cells became flattened. The cells were no longer densely packed together, while contact remained between adjacent cells each was more diffuse than previously (Figure 5.4.2.3)

Substrate	Total number of ganglia observed	Comments
No coating	3	Ganglia attach to the coverslip. Some axonal outgrowth and scattered Schwann cells. EGFP fluorescence remains in the ganglia and surrounding area
Collagen only	3	Outgrowth of axons seen. Sparse Schwann cells from this. EGFP expression remains at comparable levels to previously in the ganglia
Laminin Only	4	Ganglia adhered to substratum. Proliferation but little outgrowth. Fluorescent cells were seen within the ganglia. No alteration in the relative level of expression.
Poly-lysine only	3	Little axonal proliferation from ganglia, sparse Schwann cell numbers. EGFP expression prominent.
Matrigel	4	Ganglia adhere to substrate. Extensive axonal and Schwann cell outgrowth. Cells not so dense as seen on collagen/poly-lysine. Apparent reduction in the level of EGFP expression. Restriction of expression remains.
Collagen/Poly-lysine	3	Ganglia adhere successfully. Complete axonal and Schwann cell outgrowth. Fluorescence levels as previously

Table 5.4.2.1 Summary of the behaviour of ganglia following replating on different extracellular matrix components.

Figure 5.4.2.2 Fluorescence of the Transgene Remains in Ganglia Plated on a Range of Substrates. Dorsal root ganglia adhere poorly to alternate substrates and subsequent Schwann cell outgrowth is affected. **A and B.** When plated on a substrate of collagen the ganglion adheres and limited Schwann cell proliferation accompanies this, as is evident in the phase contrast image. A number of EGFP positive cells are seen within the ganglia as indicated by arrows when illuminated with excitatory light. **C and D.** Adhesion and some outgrowth of Schwann cells are seen when ganglia are re-plated on a laminin. Positive cell bodies with elongated processes are seen within the ganglion. **E and F.** Ganglia on the substrate poly-lysine show poor neuronal outgrowth and Schwann cell division. However expression of the transgene remains in positive Schwann cells. (A, C and E phase contrast optics). Scale bars A,C and E 200μm, B,D and F 100μm.

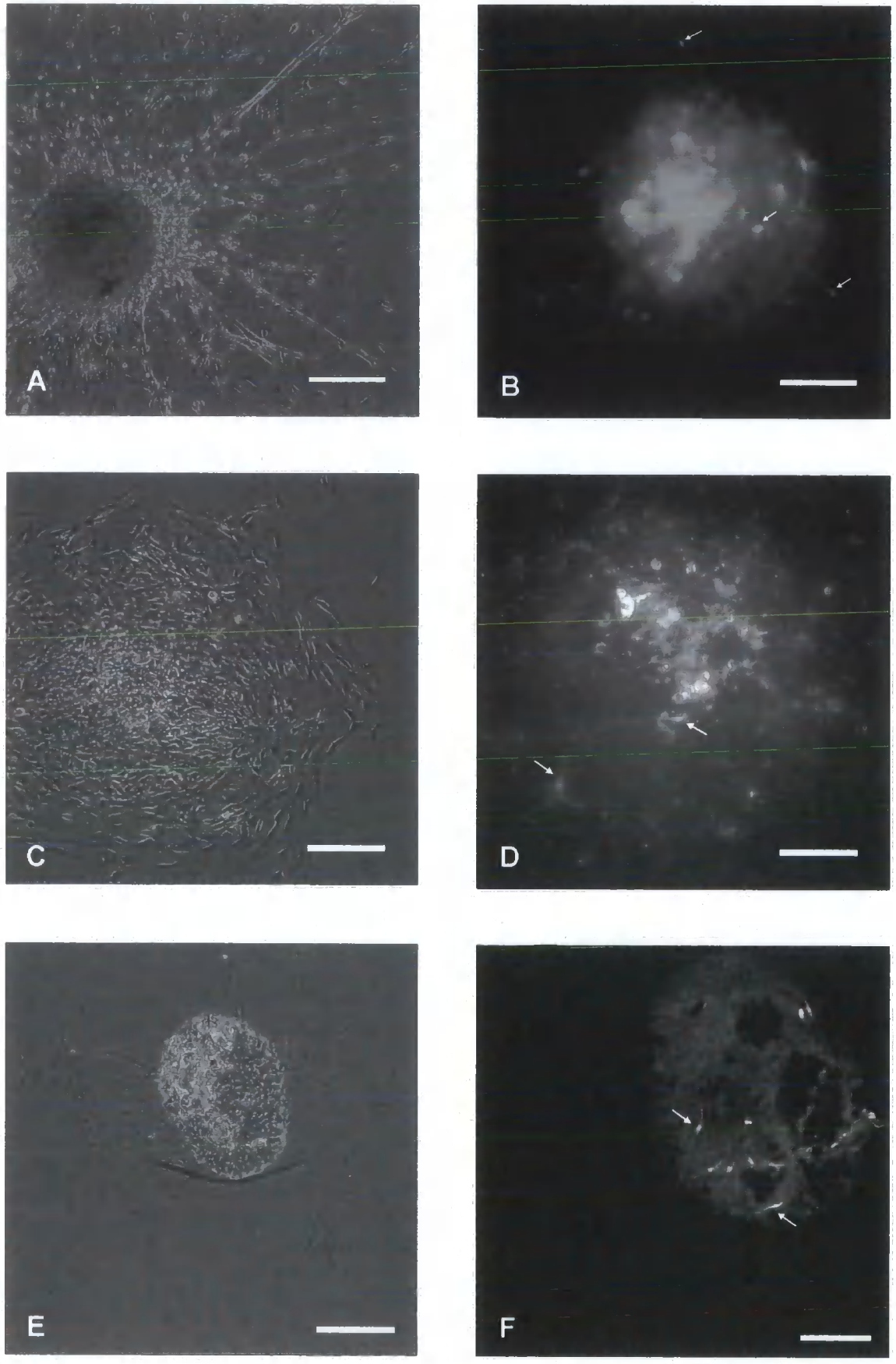
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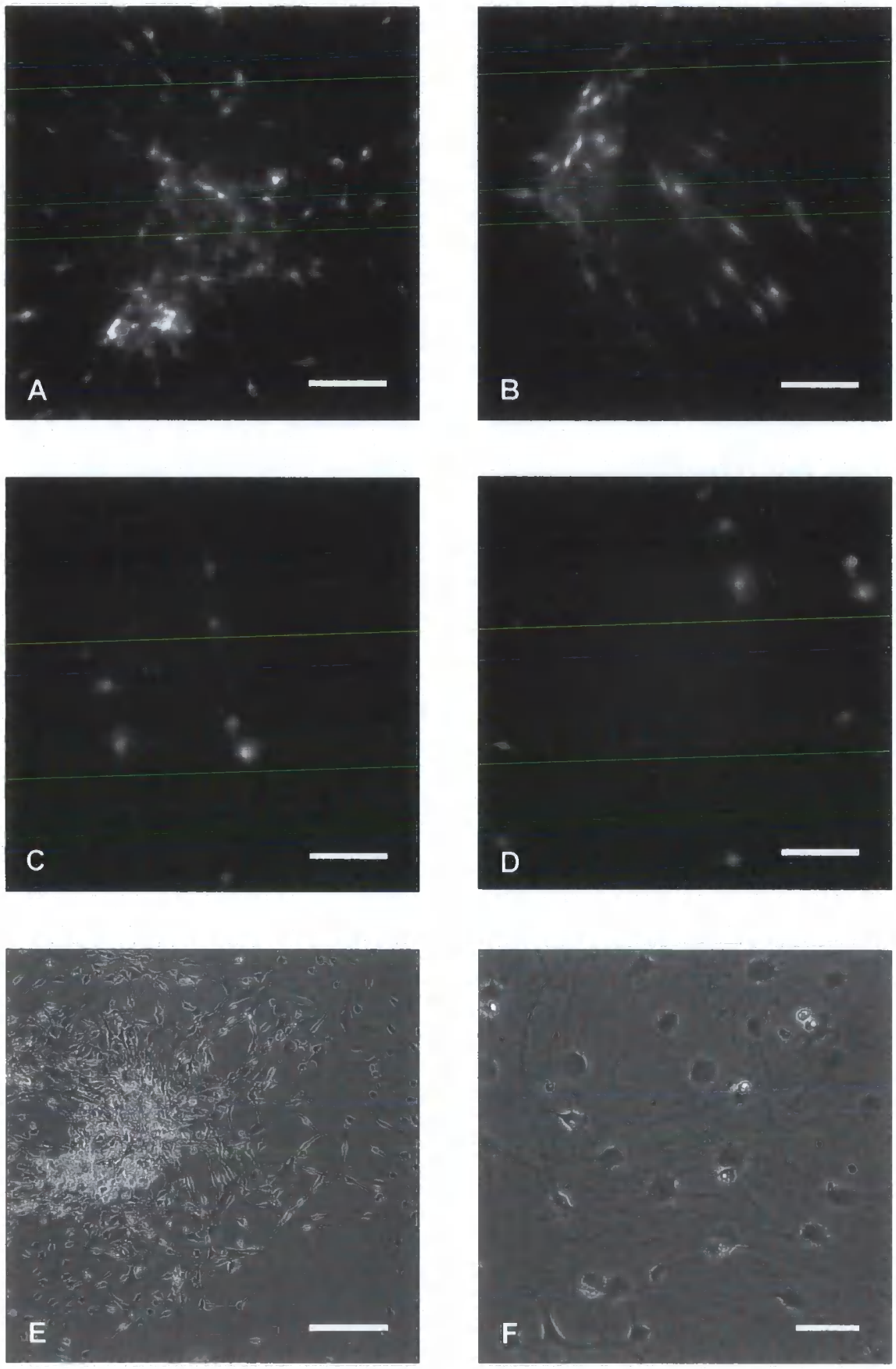
Figure 5.4.2.3 The Morphology of the Schwann Cells and the Pattern of EGFP Expression is Altered in Ganglia Plated on the Substrate Matrigel. The expression pattern of the transgene was investigated on a number of different substrates. **A and B.** Prior to re-plating the ganglia were assessed for the extent of expression on the normal substrate collagen/poly-lysine. **C and D.** Following re-plating on matrigel the morphology of the Schwann cells is greatly altered. The cells are now smaller and flattened. Many do not extend processes. In addition the observed level of fluorescence appears lower than that seen before the procedure was carried out. Scale bar 100μm. **E and F.** Under phase contrast optics the morphology of the Schwann cells can clearly be seen. Although some remain stellate with higher magnification the flattened shape of the cell body surrounding the nucleus and the extended cytoplasm can be seen. Scale bar 200μm and 50μm respectively.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Independent visual assessment of the level of fluorescence seen in Schwann cells replated on the Matrigel substrate indicated that the proportion of cells expressing the transgene appeared reduced. In addition the intensity was also diminished, although this may have been due to the increased area of the Schwann cell cytoplasm brought about by the change in morphology.

At no time was fluorescence induced in Schwann cells from cultures of embryos that were positive for the transgene but had not expressed EGFP before re-plating.

5.5 Summary

Comprehensive observation of the peripheral nerves of transgenic mice from neonatal day 5 (P5) to adult failed to provide evidence for the expression of the transgene *in vivo* following birth. Investigation also showed that many embryos in which the transgene was present in the genome failed to give rise to fluorescent Schwann cells in culture.

The influence of axonal contact and extracellular matrix components on the expression of the transgene and the restricted distribution of the positive cells was considered. The removal of axonal contact was not sufficient to alter the expression pattern of the transgene in any way. The restricted outgrowth of neurons and Schwann cells when plated on a number of components hindered the observation of expression within these cultures. That extracellular matrices can influence Schwann cells was demonstrated by the altered morphology of Schwann cells when plated on the matrigel substrate. The change in morphology appears also to be accompanied by a downregulation in the expression of the transgene by a number of Schwann cells.

Chapter 6.

Discussion

Chapter 6: Discussion

6.1 Summary

The P1 promoter has been shown to drive the Schwann cell specific increase in PMP-22 expression associated with myelination *in vivo*. In this study the P1 promoter was isolated from mouse genomic DNA, and was then successfully incorporated into a plasmid vector to drive the expression of the enhanced green fluorescent protein. The cell specificity of the construct was demonstrated in a series of functional studies. The expression of the transgene was seen in both primary Schwann cells and the Schwann cell line SCL4.1/F7. In addition EGFP expression was demonstrated in diverse cell types associated with the non-neural expression of PMP-22. In an unrelated cell type, the COS-7 cell line, no expression was observed.

The Hind III/Afl II fragment of the construct that contains the promoter and reporter gene with regulatory regions was microinjected into pre-implantation fertilised embryos. Of 240 viable embryos that were re-implanted into pseudopregnant recipients 50 developed to term, and of these 3 transgenic founder lines have been found that transmit the transgene through successive generations. Variation in copy number between them was evident by Southern blot analysis.

Analysis of embryos from founder line four showed expression of the transgene in a Schwann cell specific manner with non-neural expression seen in the skeletal muscle, lung and liver. The Schwann cell expression was mosaic, not all Schwann cells from any one mouse were positive for EGFP expression. Comprehensive observation of the peripheral nerves of transgenic mice from neonatal day 5 (P5) to adult failed to provide evidence for the expression of the transgene *in vivo* following birth. Investigation also showed a further level of variation as many embryos in which the transgene was found to be present in the genome by PCR failed to give rise to fluorescent Schwann cells in culture.

The influence of axonal contact and extracellular matrix components on the expression of the transgene and the restricted distribution of the positive cells was considered. The removal of axonal contact was not sufficient to alter the expression pattern of the transgene in any way. The restricted outgrowth of neurons and Schwann cells when plated on a number of components hindered the observation of expression within these

cultures. That extracellular matrices can influence Schwann cells was demonstrated by the altered morphology of Schwann cells when plated on the matrigel substrate. The change in morphology appears also to be accompanied by a downregulation in the expression of the transgene by a number of Schwann cells.

6.2 Possible Causes of the Observed Variation of Expression

The use of transgenic mice has been extensive since the technique was first developed. It is common to see variation of expression between different lines of animals microinjected with the same DNA fragment. Even with transgenic animals previously created to study myelin proteins there has been reported mosaicism. For example the expression of MBP promoter driven lacZ in transgenic mice was absent from most adult oligodendrocytes (Foran and Peterson, 1992). The rescue of the shiverer phenotype by the introduction of a functional myelin basic protein gene was also mosaic (Popko *et al.*, 1987; Readhead *et al.*, 1987). In addition the expression of antisense MBP cDNA caused variation between cells in the expression of endogenous MBP (Katsuki *et al.*, 1988). Enhancer and repressor elements that regulate the expression of a particular gene can exert their influence from a great distance. Problems of expression have often been thought to be due to an absence of important 5' regulatory regions.

Stable positional effects have also been considered to determine differences of expression between lines. The integration of an equivalent transgene within different sites can effect the resulting level of expression. A number of studies have described transgenic lines in which variation in the level of expression is seen both between lines and within individuals of the same line indicating some other method of restriction. Palmiter *et al.* (1984) produced a mouse in which the levels of expression of herpes simplex virus thymidine kinase varied by more than an order of magnitude between progeny of the same line. *Dobie et al.* (1996) investigated the expression of the β-lactoglobulin gene in the mammary gland of transgenic mice and found variegation of expression, only a proportion of the cells expressed the transgene. Studies in Drosophila and yeast have provided insights into the mechanism of transgene silencing that can also be applied to mice.

6.2.1 Potential Influences on Expression Variation

6.2.1.1 Multiple Insertion Sites

A number of other possible factors that could influence the expression of the transgene must be considered. Differential expression in transgenic animals may be the result of multiple sites of insertion of the foreign DNA. For example if a founder animal has integrated a transgene at two sites and only one of these is active then depending upon which set of tandem repeats the offspring inherits, it may or may not show EGFP expression. This would be evident by discrepancies in the copy number between the founder and the expressing and non-expressing offspring.

Further indication of multiple insertion sites would be evident by the restriction pattern obtained by Southern blot analysis. If different offspring inherit copies from different chromosomes the junctional fragments generated following digest of the genomic DNA would be different.

In my mouse model an accurate determination of the copy number of the mice cannot be made at this point although there is no obvious gross difference in the copy number between individuals. To enable a precise comparison Southern blots would need to be hybridised with an internal probe of an endogenous gene. This could be used to equalise the amount of DNA loaded into each lane and correct for any discrepancies in the transfer process.

The restriction pattern between offspring that have the transgene but differ in their expression of it gave identical bands on the Southern blot and appears the same as that of the founder. However the additional fragments were seen more strongly in the offspring than the founder animal. This is likely to be due to technical differences such as the quality and quantity of DNA, discrepancies in the blotting procedure or the activity of the probe. The restriction pattern is consistent both between individuals of the same litter and between litters.

The additional bands are likely to correspond to junctional fragments, regions that lie between the Bam HI site of the terminal tandem repeats and the next Bam HI site within the genomic DNA on either side of the insert. Specifically these fragments are restricted to the DNA of positive animals and are therefore not non-specific bands that have hybridised to the probe or sections of the endogenous PMP-22 P1 promoter.

It cannot be ruled out that subtle differences in the molecular weights of the restriction bands occur and that the resolution of the agarose gel masks this. The use of alternative restriction enzymes would address this problem. It is unlikely that with a range of enzymes the fragments isolated from genomic DNA would be equivalent across the mice.

6.2.1.2 Sex Related Expression.

A further obvious difference that may give rise to distinct populations of transgenic mice is their sex. As in humans, the presence or absence of the Y chromosome determines the sex of a mouse. Females lack the Y chromosome and have two copies of the X chromosome. In order to maintain equal dosage of genes expressed on the X chromosome between the two sexes one is inactivated in female mice. If a transgene inserts on the paternal copy of the X chromosome that is then inactivated in an individual female offspring it will be unable to express the transgene.

Alternatively if the transgene has incorporated into the Y chromosome then only the male offspring can inherit it. However this would not explain in the present study the discrepancy of the animals that possessed the EGFP gene in their genome yet did not express it.

Moreover table 5.3.2 describes the number of DRG co-cultures studied over a six month period. Often within a litter while a number of embryos had the transgene in their genomic DNA none of them expressed the EGFP protein. It is unlikely given the number of cultures studied that all of the mice that had the transgene and did not express it were of the same sex. Conversely, the infrequency with which mice express the transgene makes it unlikely that the determining factor is the sex of the animal. Equal numbers of male and female offspring would be expected, yet the figures for animals that express the transgene do not reflect that.

The sex of a mouse can be determined at the embryonic stage. At the E15 developmental stage the gonads of each sex can be found in a position relative to the kidneys. The male testes appear round in shape and at this stage in development are found deeper in the pelvic region that the female ovaries. The ovaries are more ovoid in nature and are

partially enveloped by the paramesonephric duct. The tissue appears "spotty" when light is shone through it.

Identification of the presence the Y chromosome would also prove an indication of the sex of the individual. This could be achieved by amplification of a gene that is found upon the Y chromosome by PCR. For example the gene for a male specific histocompatibility antigen that is found on the Y chromosome gives a distinct restriction pattern in a Southern blot between male and female (Greenfield *et al.*, 1996). The mRNA sequence for this protein is readily accessible in the Genbank database (accession number Y09222) and primers designed against it would allow identification of mice that have the Y chromosome.

To determine the influence that the sex of a mouse has on the expression of the P1-EGFP transgene, transgenic embryos could be analysed to determine their sex on dissection. In addition genomic DNA was extracted from embryos following a number of previous dissections. Suitable oligonucleotide primers could be employed with an appropriate PCR protocol to utilise this resource.

6.2.1.3 Growth Arrest

The regulation of PMP-22 and its alternate promoters in response to growth arrest has been extensively studied. The position through the cell cycle of individual cells within culture may be determining the expression of the transgene. While this possibility has not been investigated observation suggests that this is not the cause of the variability of expression.

The relative level of expression within an embryo can be estimated as the ganglia are removed. If one embryo has low numbers of positive cells immediately following dissection in comparison to another, then subsequent time in culture will not alter the relative levels between the two. This suggests that the proportion of expressing cells is determined independently of tissue culture conditions.

Within the DRG co-culture system Schwann cells are highly proliferative expanding throughout the culture. It is possible that a number of these are in a state of growth arrest, under which conditions it is suggested that PMP-22 is upregulated. However the variation in proliferation between cultures derived from individuals within the same

litter, maintained in identical culture conditions, is unlikely to be as great as the difference often observed between them in the levels of expression.

The growth state of a cell can be determined by the nature of the molecules that are expressed at the nuclear membrane. For example in DRG co-cultures from transgenic embryos immunocytochemistry could be used to determine the presence of specific nuclear lamins associated with cell division at the nucleus of expressing cells. The incorporation of Bromodeoxyuridine (BrdU) into actively dividing cells, followed by their identification through antibody labelling of the BrdU would provide an alternative method of distinguishing between the growth states of cells.

The maintenance of Schwann cells in low serum conditions (0.5% FBS) has been used to induce growth arrest. If these conditions were to be imposed upon the co-cultures any alteration in the levels of expression may indicate a role for growth arrest in the expression of the transgene.

6.2.1.4 Position Effect Variegation

Work in *Drosophila* has shown that the position of a gene within different regions of the chromosome can affect the expression that is seen. Within eukaryotic cells the chromosomal material can take on a number of forms. Heterochromatin, found at the centromere and telomere of a chromosome appears deeply stained and condensed throughout the cell cycle and is relatively gene poor. By comparison euchromatin appears diffuse during interphase and is rich in functional genes. It was found that if a gene was incorporated into a region of heterochromatin this brought about conformational change over the transgene that prevented interaction with the transcriptional machinery and so silenced the gene. Variation between individual cells is accounted for by differences in the extent of linear extension of the heterochromatin region (Martin and Whitelaw, 1996; Henikoff, 1998).

The nature of the transgene also influences the silencing effect. DNA fragments tend to insert as linear head to tail arrays. It has been shown that arrays with high copy number tend to be focal points of heterochromatin formation. In Drosophila as few as three copies of a transgene have been seen to display a position effect variegation like

phenotype (Dorer and Henikoff, 1994). Garrick *et al.* (1998) investigated this effect in mice. A construct containing the β-globin promoter region and the *LacZ* reporter gene was used to produce multiple transgenic mouse lines. In addition the transgene incorporated a 34 base pair loxP site, the recognition site of the Cre recombinase enzyme of bacteriophage P1. The transgenic lines derived from this series of microinjections were examined for the expression of β-galactosidase in erythrocytes. Two transgenic lines were identified that displayed variable expression combined with a copy number of over 100. Oocytes derived from these mice were microinjected with a circular plasmid that possessed the Cre recombinase gene. This mediated recombination between loxP sites within the transgene array and removed the intermediate DNA as a circular episome. The copy number in these lines was therefore reduced. Analysis of the transgene expression following the reduction in copy number, indicated a strong increase in expression in comparison to the founder line. Since the site of insertion remained the same the high copy number was thought to have been the cause of the restricted expression. Subsequent studies showed that the transgene in the founder line was more resistant to DNase digestion than that in the mice with reduced copy number. This suggested that homolgous pairing between monomers within the transgene array were forming heterochromatin structures that were repressive to the expression of the transgene.

The degree of variation was also found to be dependent upon other factors. It has been shown that age can effect the expression pattern. Robertson *et al.* (1996) found that among a line in which the level of variation of expression remained constant between offspring the developmental stage at which the reporter gene assay was carried out was significant. Compared to embryonic stages at later ages the proportion of cells that expressed the transgene was reduced.

The influence of position effect variegation (PEV) can be overcome by other *cis* acting factors. Locus control regions (LCR) direct high levels of tissue specific expression of transgenes independent of the site of insertion. They act to open chromatin domains and allow *trans* acting factors access to a gene (Festenstein *et al.*, 1996). The LCR of the human CD2 gene has been identified and deletion studies have demonstrated the need

for a complete LCR to achieve high and non-variegated expression even when the gene is shown to be located in a centromeric region (Festenstein *et al.*, 1996).

The P1-EGFP transgenic mouse shows mosaicism of expression. Within an individual animal not all Schwann cells show EGFP expression and within individuals of the same litter the extent of the variation is different. If this were a stable position effect, with the surrounding genetic environment silencing expression it would be expected to do so consistently in all animals since they share the same site of insertion. However position effect variegation describes a situation in which variation occurs between mice of the same founder line, more accurately describing this mouse line.

6.2.1.5 Fluorescence In Situ Hybridisation

A valuable experimental tool that could illuminate many of the aspects that determine variegation is the use of fluorescence in situ hybridisation or FISH. This method utilises DNA or RNA probes that bind to specific sequences within the genomic DNA that can then be visualised by incubation with a fluorescent label. This allows identification of the site of that gene to a specific region of an individual chromosome. Cells obtained from easily accessible tissues or blood and are swollen hypotonically and then fixed on slides. The DNA is denatured and fixed in ethanol to prevent reannealing. It is then incubated with the specific probe labelled with reporter molecules such as biotin and allowed to hybridise. Following washing the slide is then incubated in fluorescent reagents such as fluorescent labelled anti-biotin antibody. The location of the transgene insertion site can be seen using fluorescent microscopy techniques, and combined with banding of the chromosomes the site can be identified (Trask, 1991).

This technique could clarify many issues regarding the behaviour of the transgenic mouse. FISH using an EGFP probe against the chromosomes of the founder mouse would identify the chromosomal site of insertion, it would be evident if more than one insertion site existed. Also through recognition of the chromosome in which it has inserted the issue of X-linked inactivation could be determined. Comparison of FISH results from transgenic F_1 litters that display differential expression of the EGFP protein, could rule out the occurrence of gene rearrangements as the cause of the variation in

variation in expression. Once these possibilities have been disproved the issue of position effect variegation seems the likely cause of the discrepancy of expression of the transgene. FISH can also determine the position of the transgene within any given chromosome. The incorporation of the foreign gene into the centromere or teleomere regions that are rich in heterochromatin could be indicative of this outcome.

6.3 Detailed Studies of the Transgenic Mouse Line 4

Despite the variable expression pattern of the transgene within the mouse line a series of experiments were performed to determine the regulation of the P1-EGFP construct within the transgenic line.

6.3.1 Expression of the Transgene in the Target Cell Type.

The ability to visualise EGFP expression in live Schwann cells provides an advantage over other methods of recognition. This system would allow the observation of the migration and interaction of non-myelinating Schwann cells with neurons against a clear background as only a proportion of these Schwann cells express the transgene. With changed culture conditions the progression of the Schwann cell as myelin formation is initiated and proceeds could be monitored, giving a dynamic visual display of myelination *in vitro*.

Transgenic line four has been extensively studied *in vitro*. The preparation of co-cultures from the dorsal root ganglia of E15 embryos allowed immunostaining and morphological identification of positively expressing cells as Schwann cells.

The expression of the transgene could be seen within the ganglia at the time of removal from the embryo. This indicated the transcription of PMP-22 through the regulation of the P1 promoter at embryonic day 15 or at some point prior to this time. Supporting evidence for the expression of PMP-22 in glial cells at this embryonic stage was found in the expression of EGFP in cells morphologically similar to Schwann cells, cultured from the skeletal muscle of a transgenic embryo. The crude nature of the dissection

technique means that it is possible that sections of the sciatic nerve were included in the preparation.

Baechner *et al.* (1995) found PMP-22 expression in other cells of neural crest origin at this time and the myelin protein P_0 is also expressed in Schwann cells during development. P_0 expression is seen in Schwann cell precursors and at birth the levels of this protein are down-regulated in non-myelin forming Schwann cells and up-regulated in myelin forming cells (Lee *et al.*, 1997). PMP-22 and P_0 show strong correlation of expression during myelination (Kuhn *et al.*, 1993; Notterpek *et al.*, 1999). In mature myelin P_0 forms homophilic interactions between molecules of opposing layers and maintains the compact nature of the sheath. An association between PMP-22 and P_0 has been shown *in vitro.* Cells transfected with P_0 were co-cultured with cells expressing PMP-22. Immunostudies showed that the two proteins co-localised at the opposing membranes of adjacent cells (D'Urso *et al.* 1999). The similarity of expression and the interaction between the two at this time is thought to demonstrate a functional significance of this relationship in compact myelin. It may be that this extends to the embryonic expression of both proteins in the Schwann cell precursor.

The possible role of PMP-22 at this time is unknown. Expression of P_0 in neural crest cells is thought to be the earliest marker of the glial cell lineage. Its embryonic expression, suppression in non-myelin forming cells and its appearance following nerve injury has lead to the suggestion that it has a role in development and regeneration that is independent of myelination (Lee *et al.*, 1997). PMP-22 has been suggested as a modulator of the cell cycle and one aspect of its expression is thought to be that of a growth arrest specific protein. However at this point in development Schwann cell proliferation rates are high as numbers are matched to those of axons. In addition investigations have suggested that it is the P2 and exon 1b transcript that are associated with growth arrest whilst P1 and exon 1a are involved in the myelination process (Bosse *et al.*, 1994; Zoidl *et al.*, 1995).

6.3.2 Non-neural Expression of the Transgene.

The organs of three transgenic E15 embryos were assessed for the expression of the transgene. In only one instance in which the expression levels in the DRG co-cultures of the individual were high was non-neural expression seen.

Non-neural expression of PMP-22 in the lung, liver, heart and skeletal muscle have been reported for the adult mouse (Spreyer *et al.*, 1991; Welcher *et al.*, 1991). During early organogenesis at 11.5 days pc high expression was seen in the capsule surrounding the liver. At 14.5 days p.c. or E14.5, expression was seen in the cartilage of the forming vertebrae. By E16.5 the neural crest derived connective tissue and skin of the face were expressing PMP-22. Parts of the skeletal system including the femur and vertebrae, muscle tissue such as the diaphragm and intercostal muscles, mesenchymal cells of the lung, all show PMP-22 expression at this time. No expression was reported in the heart or liver (Baechner *et al.*, 1995).

Morphological considerations suggest that the expression seen in the skeletal muscle culture of the P1-EGFP embryo was restricted to Schwann cells from contaminating sciatic nerve. Immunolabelling with the Schwann cell specific S-100 antibody would demonstrate this.

A small number of cells in lung and liver cultures were also positive for EGFP expression. These cells had a flattened morphology with large cytoplasmic areas. Subsequent analysis of these tissues may provide insight into alternative roles of PMP-22. Previous studies of non-neural expression have utilised the whole organ and were unable to identify which individual cells were expressing EGFP. The fixation and sectioning of tissues such as the liver and lung from embryonic or adult transgenic mice may enable identification of the site of expression within each tissue. Determination of the cell type and lineage of positive cells may further illuminate the significance of PMP-22 expression at these sites.

6.3.3 Corroboration of the Transgene Expression with that of Endogenous PMP-22

To ensure that the expression observed in E15 Schwann cells and embryonic organs genuinely reflects expression of PMP-22 further studies must be carried out. Reverse transcriptase PCR could be used to detect any PMP-22 exon 1a containing transcripts within the entire population of cells. A modification of the procedure in which total RNA is isolated from a single cell could be used to specifically identify expression within EGFP positive cells. Alternatively expanded co-cultures or ganglia on removal from the embryo could be fixed and immunolabelled with a PMP-22 specific antibody. Incubation with a fluorescent rhodamine secondary antibody would allow simultaneous identification of cells that express the PMP-22 protein and those that show EGFP fluorescence.

6.3.4 Validation of the P1 Promoter

The initial analysis of the transgenic mouse line four indicates that the 1.8kb region of the P1 promoter (Bosse *et al.*, 1999) is sufficient to regulate the tissue specific expression of a reporter gene. Previously 3.3kb of 5' sequence to exon 1a have been used to drive expression both in Schwann cells and fibroblasts with limited promoter activity (Suter *et al.*, 1994).

The regulation of other myelin proteins has also been investigated through the production of transgenic mice in which specific promoters were used to drive reporter gene expression. The myelin basic protein (MBP) promoter has been extensively studied, and a number of transgenic lines have been produced with different lengths of the 5' untranslated region regulating gene expression (for review Ikenaka and Kagawa, 1995). As little as 256 bp of the MBP promoter region has been shown to direct oligodendrocyte specific expression of reporter genes (Miura *et al.*, 1989; Goujet-Zalc *et al.*, 1993). Distinct Schwann cell expression however has not been seen with any construct implying the absence of an important regulatory region, although some expression was reported when up to 6kb of 5' sequence was used (Gow *et al.*, 1992; Foran and Peterson, 1992; Friedrich *et al.*, 1990).

6.3.5 Attempts to determine the regulation of the transgene during development.

Within the DRG co-culture system employed in this study not all Schwann cells from an individual transgenic mouse expressed the EGFP protein. This may have been due to the developmental stage at which the Schwann cells were removed from the influence of *in vivo* signals. During post-natal development there is a great rise in the transcription of PMP-22 from the P1 promoter (Suter *et al.*, 1994). It was hypothesised that if the transgene considered here was regulated correctly in a manner equivalent to that of PMP-22, examination of peripheral nerves from neonatal animals would show that expression of the transgene would increase during this time. The sciatic nerve was therefore removed from a series of animals, each identified as possessing the transgene by PCR analysis, and observed by confocal microscopy. In all animals from all founder lines and at ages ranging from postnatal day 5 to adulthood no expression of the construct was identified. In the DRG co-culture system embryos were also identified as positive for the transgene but that did not show EGFP expression.

Southern blot analysis eliminated the possibility of contamination of the PCR reactions and also highlighted that the restriction pattern of all the offspring was the same, and no gross difference in the copy number was obvious between them.

The site of the regulation of transgene expression may be further investigated through reverse transcriptase PCR to identify the presence or absence of EGFP mRNA in the co-cultures of positive animals that do not show fluorescence. In addition a section of sciatic nerve from the animals used in the study of neonatal expression was embedded in Tissue-Tek and frozen at –80°C. mRNA material could be removed from these nerves and assessed for the EGFP transcript.

6.3.6 Analysis of the Transgene *In Vitro*

6.3.6.1 *In Vitro* Myelination

If a mechanism similar to PEV is the cause for the variation of expression seen within the P1-EGFP transgenic mouse then there is no way to predict the likely expression pattern of any individual. This will restrict analysis of the regulation of the P1 promoter region *in vivo*. However the E15 DRG co-cultures can be induced to myelinate if maintained in the appropriate media. Commonly, following the initial dissection the co-cultures are pulsed with anti-mitotic reagents to remove proliferating fibroblasts and Schwann cells. This results in a pure culture of DRG neurons. Concomitantly co-cultures of transgenic, expressing Schwann cells are allowed to expand and the neurons are then removed to leave a pure population of Schwann cells. These can then be trypsinised from the culture dish and re-plated at a suitable density onto the pure neurons. The cultures are first maintained in defined medium that allows neuron stimulated proliferation of the re-seeded Schwann cells. It is then replaced with myelinating media, basal media with 10% FBS and 50μg/ml ascorbic acid. Within 7 days myelin formation will begin and the cultures can be maintained for a number of weeks.

In vitro myelination can be difficult to achieve consistently. The discovery of the influence of progesterone on both myelin formation and individual myelin protein expression has lead to culture methods that can increase that efficiency (Notterpek *et al.*, 1999).

During injury of the peripheral nerve levels of progesterone remain high. The addition of progesterone inhibitors at this time impedes the subsequent remyelination of regenerating nerves that as a result show decreased myelin thickness. Myelination can be enhanced by the addition of exogenous progesterone with the sheath showing an increased number of lamellae. The overall percentage of myelinated fibres is not altered with varying levels of progesterone, so it is suggested that it is not involved with the initiation of myelin formation, but stimulates the ongoing production of myelin (Koenig et al., 1995).

The addition of progesterone to myelinating media has been shown to enhance the efficiency of myelination *in vitro* (Notterpek *et al.*, 1999). It was found that this stimulated the expression of the myelin proteins MBP, P_O and PMP-22 in Schwann cell co-cultures (Desarnaud *et al.*, 1998; Notterpek *et al.*, 1999).

One advantage of the variation of expression among cells of one individual is that the full extent of an individual Schwann cell can be seen. The fine processes that extend from the Schwann cell body can be difficult to distinguish fully in dense three dimensional cultures. The absence of background fluorescence and the intensity of the EGFP signal enables the limits of a single Schwann cell to be identified. Confocal microscopy techniques could be used to follow the behaviour of a single Schwann cell over time and in a three dimensional manner to give clear and precise evidence of the manner in which the Schwann cell and neuron interact at this time.

It would also be interesting to observe the expression of the transgene in other Schwann cells as the P1 promoter is used more extensively as myelination proceeds. Although it would be difficult to quantify an increase in EGFP expression visually within an individual cell a series of semi-quantitative single cell RT-PCR reactions could monitor an increase in EGFP mRNA expression at different stages of the myelinating procedure.

The expression of EGFP in Schwann cells at E15 may not represent the entire population that is able to do so. The initiation of myelination may induce EGFP expression in previously non-expressing Schwann cells. This may be a distinction between different stages of development among the population *in vitro* alternatively it may represent a novel role for PMP-22 in a population of Schwann cells that is independent of myelination. The immunostaining experiments that were suggested in section 6.3.3 would address this issue. If the number of EGFP positive cells remains constant and PMP-22 immunolabelled cells are identified that do not express EGFP then the initial population of positive cells represents the extent of expression within that individual.

Some evidence has been obtained that the expression of the transgene can be influenced through study of the interaction of the non-myelinating Schwann cells with the extracellular matrix and the DRG neurons. The influence of both neurons and the extracellular matrix has been shown to have a profound effect on the expression of myelin proteins and the behaviour of Schwann cells. In an attempt to determine how influential these factors were on transgene expression in the Schwann cell they were disrupted and altered expression was searched for.

6.3.6.2 Neuronal Influence on Expression

Co-cultures immunolabelled with anti-neurofilament antibodies clearly showed the great extent of neuronal outgrowth across the tissue culture dish indicating that restricted axonal outgrowth was not underlying the low expression seen.

Following the removal of the dorsal root ganglia from the co-cultures the remaining neuronal processes were shown to degenerate within 48 hours. Over time the loss of the neurons affected the morphology of the Schwann cells, however no decrease in expression was seen. That expression can continue without axonal presence was reiterated by the observation of potential fluorescent Schwann cells in the skeletal muscle culture. Due to the method of dissection axons in the sciatic nerve would have been severed from their cell bodies and would subsequently degenerate.

It is unlikely that the persistent EGFP signal following axonal degeneration is a reflection of a stable population of EGFP protein. Clontech state the half-life of EGFP as twenty four hours and so it should prove dynamic enough to reflect changes in expression over such a course of time examined here. Removal of the neurons from the co-culture at an earlier stage and maintenance of the culture over longer periods of time should solidify the validity of this observation.

The restricted distribution of the positive cells did not alter after the removal of axons.

These observations suggest that the expression of EGFP in this population of Schwann cells was independent of axonal contact and myelination and that axonal influences did not restrict the expression of the transgene.

Schwann cells have been shown to be dependent upon many axonal signals. Precursor Schwann cells are dependent upon neuronal signals for survival, axonal contact is required by mature Schwann cells for the formation of myelin (Jessen *et al.*, 1994; Lemke, 1996). PMP-22 was originally described as a protein that was differentially expressed following nerve crush (Spreyer *et al.*, 1991; Welcher *et al.*, 1991). Axonal degeneration causes downregulation of the expression of PMP-22, that recovers as regeneration and remyelination occurs. Specifically it was demonstrated that the myelin specific expression of PMP-22 was restricted to the exon 1a containing transcript that is driven by the P1 promoter (Suter *et al.*, 1994).

However PMP-22 has been demonstrated in the cytoplasm of non-myelinating Schwann cells in co-culture with neurons. In myelinating co-cultures, MAG negative Schwann cells have been identified in which PMP-22 expression was found at the plasma membrane, this translocation of the protein is not normally seen in cells that are not forming myelin. And in addition MBP negative Schwann cells of the rat sympathetic tract have also been found to express PMP-22 (Notterpek *et al.*, 1999). This indicates that neuronal contact and myelination do not strictly regulate the expression of the protein. However it does not preclude the possibility that neuronal and extracellular influence are still required at some level.

It is possible that a neuronal signal is required to initiate expression at an earlier developmental stage. DRG dissection from embryos younger than E15 becomes progressively more difficult as the tissue becomes softer. In situ hybridisation of whole younger embryos with an RNA probe against EGFP may allow determination of the age at which expression begins this may then be related to the interactions that are occurring between axons and Schwann cell progenitors at that time. It may also be compared to the expression of the myelin protein P_O during development to determine the extent of their interaction at this time.

6.3.6.3 Extracellular matrix regulation of Transgene Expression

Extracellular matrix products have been shown to have a profound effect on the differentiation of the Schwann cell. Once Schwann cells have associated with axons and in the presence of the necessary factors the Schwann cell secretes a basal lamina before myelination proceeds. This extracellular matrix polarises the cell and allows myelination to continue (Bunge *et al.*, 1986). The role of the extra cellular matrix in the initial stages of myelination that accompany changes in gene expression have been discussed in detail in section 1.3.2.

Dorsal root ganglia co-cultures were maintained in a chemically defined media that was not permissive for the formation of a basal lamina due to the absence of serum and ascorbic acid. However Schwann cells have been demonstrated to secrete some extracellular proteins independent of axonal contact and myelin formation. Punctate labelling of laminin and heparin sulfate proteoglycan is seen in Schwann cells in non-myelinating media, however they are not organised into a functional basal lamina (Cornbrookes *et al.*, 1983). Furthermore since these factors are secreted locally by individual Schwann cells an attempt was made to investigate its role in restricting the expression of the transgene. The ganglia were plated on a number of substrates, however limited growth of the cultures prevented any direct comparison of expression levels and patterns before and after re-plating. On the Matrigel substrate successful culture was observed. This was shown to greatly affect the morphology of the Schwann cell and some evidence was found for a reduction in the number of Schwann cells that expressed EGFP. However the restricted regions of expression remained.

Matrigel is a soluble basement membrane extract from the Engelbreth-Holm-Swarm tumor. Its major components are extracellular matrix proteins; laminin; collagen type IV; entactin and heparin sulfate proteoglycan. However these proteins are not organised into a functional matrix.

The importance of the extra cellular matrix in determining the morphological shape of a cell has been demonstrated. Once a basal lamina has been established and expression of the myelin proteins begins, the Schwann cell undergoes a number of morphological changes. Undifferentiated Schwann cells with short processes and round nucleus, extend

the cellular processes and the nucleus elongates. In serum-free media conditions expression of the myelin proteins P_0 and MAG can be induced, however basal lamina formation is not. In the absence of a basal lamina the morphological alterations that normally accompany the change in gene expression do not occur (Fernandez-Valle *et al.*, 1993). The same report also found that with the removal of serum from the growth media Schwann cells in a DRG co-culture were able to express P_0. However maintenance in media with serum but no ascorbic acid resulted in suppression of P_0 levels. Subsequent addition of ascorbic acid to the media caused a rapid increase in P_0. This was argued to show that the expression of this protein is linked to basal-lamina formation.

The observation that manipulation of the extracellular matrix may be influencing the extent of the expression of the P1-EGFP transgene needs to be confirmed and expanded upon.

Ganglia plated and maintained on clean coverslips would secrete extracellular components across the surface of the coverslip. If the cells were later removed and the deposited extracellular matrix examined, localised variation in the factors secreted may become evident. Repeated cycles of freezing at –20°C followed by thawing to room temperature osmotically ruptures the cells and their debris can be removed by washing. Incubation of the coverslips with a range of different antibodies against matrix components would indicate any differences.

In a preliminary experiment a co-culture from an embryo that expressed the P1-EGFP transgene in a large proportion of the cells was allowed to deposit extracellular matrix components onto the surface of a coverslip. Following the removal of the cells by repeated freeze thawing and adequate washing the coverslip was re-plated with ganglia from cultures in which the level of expression was low. In two of the four ganglia that were replated it appeared that expression was seen in a greater number of cells than previously. This experiment needs to be repeated before any statement can be made.

The mechanisms by which the extracellular matrix could affect the expression of the construct are complex. Upregulation of endogenous PMP-22 and consequently the transgene by the induction of myelination requires exact culture conditions and is difficult to achieve. An independent method of increasing expression levels that can be achieved more readily is the addition of forskolin to culture media. Forskolin is an activator of cyclic AMP that has been shown to increase the expression of a number of myelin proteins including PMP-22 and specifically the P1 promoter driven expression of the exon 1a transcript (Suter *et al.*, 1994). The addition of forskolin to non-myelinating cultures has been shown to raise the expression of PMP-22 (Notterpek *et al.*, 1999). The exposure of the transgenic Schwann cell co-cultures to this agent will raise the expression in all cells. If an increase in the number of fluorescing Schwann cells is seen this would provide evidence that the transgene can be regulated and in a manner that is representative of the endogenous promoter.

These experiments may provide evidence for the regulation of the transgene however no mechanism is suggested by which the transgene expression is restricted. One alternative hypothesis is that in fact the restriction is not due to some physical factor that is influencing expression. The position effect variegation discussed in section 6.2.1.4 has been suggested to occur in a clonal manner (Dobie *et al.*, 1996; Festenstein *et al.*, 1996). The decision whether to express the transgene is thought to be made at an early stage in development and subsequent cells derived from that progenitor will also express the transgene. In this way the restricted expression pattern seen may reflect the existence of a small number of progenitor cells that have expanded within a localised area.

6.4 Further Suggested Study of the P1-EGFP Transgenic Line.

In the P1-EGFP mouse line four the expression of the transgene has been demonstrated in the Schwann cells of the peripheral nerve albeit in a mosaic manner. In addition the transgene was observed in a number of non-neural sites that lends weight to the argument that the exon 1a transcript while predominating in the Schwann cell also contributes to the reported non-neural expression. This is this first instance in which the

use of the P1 promoter has been attributed to individual populations of cells in these non-neural sites. Further investigation of their lineage and function may indicate potential alternative roles for PMP-22 in these cells.

The mosaic expression pattern of the transgene in line four was further demonstrated when analysis showed that not every individual that possessed the transgene within their genome expressed it despite the assumed identical site of insertion throughout the line. Southern blot analysis showed no evidence for multiple insertion sites or rearrangements of the transgene between these offspring. Other suggestions include insertion of the transgene into the silenced X chromosome. These possibilities could all be addressed by fluorescent in situ hybridisation studies investigation of the line.

The variation in expression between individuals of the same line is consistent with a mechanism similar to that of position effect variegation in which the silencing of the gene is affected by the site of insertion and the nature of the chromosomal material present at that site. The divergence between cells that express and those that do not is thought to be made early in development. If all positive cells within a given area were derived from the same progenitor this would explain the restriction of expression that is seen *in vitro*.

If the expression of EGFP in the Schwann cells at E15 as they are removed from the embryo represents a true reflection of PMP-22 transcription then it has been demonstrated that this persists independently of neuronal contact and myelination. The extracellular matrix however may influence the regulation of this expression but does not alter the restricted pattern of expression.

This transgenic mouse potentially provides an opportunity to study the influence of the extracellular matrix on the expression of the myelin protein PMP-22. It can also give rise to a novel *in vitro* system with which the myelination of axons by peripheral Schwann cells can be visualised. This could be extended to include study of the interactions between Schwann cells and neurons in the *trembler* and *trembler-J* phenotype. Point mutations within the transmembrane spanning domains of the PMP-22 protein result in abnormal myelin formation in these mice (Suter *et al.*, 1992a,b). A mating regime in which the P1-EGFP transgene is introduced into the *trembler*

background would give rise to Schwann cells that lacked PMP-22 expression with the potential to express EGFP. The rescue of such cells by adenoviral infection with functional PMP-22 cDNA would allow direct visualisation of their behaviour as they begin to express the myelin proteins and myelinate axons.

Following nerve injury the restoration of function depends upon the regrowth of surviving axons and the guidance of these neurons to their correct target with appropriate synaptic connections. In the PNS successful regeneration can occur. Neurons are able to grow through the remaining environment which includes Schwann cells, extracellular matrix components and other non-neural elements that express trophic factors and critical cell surface molecules. In addition Schwann cells can provide a permissive substrate through which CNS axons can regenerate (Aguayo *et al.*, 1990). Expansion of Schwann cells that express EGFP would provide a source of cells that would be suitable for use in transplantation studies. Injection into local sites of injury and subsequent examination of these by fluorescence confocal microscopy would allow ease of identification of the transplanted cells and aid study into their migration and behaviour in the central nervous system environment.

In conclusion while the P1-EGFP transgenic mouse line has limitations with respect to the variation of expression of the reporter gene, it remains an exciting tool with which to visualise the dynamic behaviour of Schwann cells. Further investigation of its expression may suggest potential roles for PMP-22 both during development and in Schwann cells and non-neural tissues that are independent of myelin formation.

Appendix A.

Technical Appendix

Appendix A: Technical Appendix

A1 Preparing 4% Paraformaldehyde

4g of paraformaldehyde were added to 45ml distilled water with one drop of 10M NaOH. The solution was heated to 60°C. Once dissolved 50 ml of 2x PBS were added and the solution and adjusted to pH 7.4. The final volume was made up to 100ml with dH_2O.

A2 Administration of Anaesthetics

Avertin (Aldrich). Intraperitoneal injection of 0.3 – 0.35 ml of 2.5% Avertin (per 20g mouse) produces around 20 minutes of effective anaesthetic, sub-cutaneous injection is not as effective. The lack of analgesia does not seem to have any adverse effect in the described procedures. Administration cannot be repeated as this can cause peritonitis. However there is no need for repeated use in these protocols.

Preparing Avertin for use: 2-2-2-tribromoethanol (Aldrich T484012)
Tertiary amyl alcohol (Aldrich 240486)
10g tribromoethanol + 10 ml amyl alcohol
Shake well and filter sterilise.
Dilute to a 2.5% solution in water and filter sterilise.

A3 Freezing of Microinjected Embryos for Long Term Storage.

Preservation of a transgenic mouse line can be achieved by the storage of frozen embryos that can be recovered at a later date. The Karolinska Institute (Sweden) have frozen the embryos of the MBP-EGFP mouse line until such a time as they are required. Pre-implantation embryos derived from the established transgenic line were removed from the oviducts of a pregnant female mouse. Following isolation the embryos were screened for abnormalities and healthy embryos were maintained in M2 media at room temperature.

Freezing straws were prepared; plugged at one end and loaded with 1M sucrose. An air bubble was introduced to mark the boundary between the sucrose and the embryos in the final setup.

Embryos were allowed to equilibrate in M2 media containing 1.5M propylene glycol for 15-30 minutes. Up to 20 embryos at a time were taken up and introduced into the straw. The straws were then cooled to –7°C in a cooling bath. The sucrose fraction was touched with forceps cooled in liquid nitrogen to begin cooling. Once the ice formation had spread to the propylene glycol, after a further 5 minutes the straw was frozen at a rate of 0.3°C/minute to a final temperature of –30°C. The straws were then plunged into liquid nitrogen and stored until further use.

A3.1 Thawing Frozen 8 Cell Embryos and Blastocysts

The freezing straw is removed from liquid nitrogen storage and held in the air for 40 seconds before being placed in a room temperature water bath for complete defrosting. The seal of the straw is removed and the entire contents expelled into a 35mm culture dish. After 5 minutes the embryos will shrunk considerably. They are then transfered to a dish of M2 media. Care is taken to maintain the zona pellucida surrounding the embryo intact. The embryos rapidly absorb water and resume normal appearance. Following at least three hours in culture the embryos are washed in fresh M2 media before re-implantation into the oviducts of a pseudo-pregnant recipient female mouse.

A4 Producing the pCMV-EGFP Positive Control Plasmid

A4.1 Introduction

The CMV-EGFP vector was produced for use as a positive control when investigating the expression of the tissue specific constructs. The cytomegalovirus (CMV) promoter gives rise to high levels of constitutive expression in mammalian cells and so can be used to provide positive evidence that a particular cell type has been both successfully transfected and is able to subsequently express the reporter gene.

The human cytomegalovirus is a member of the herpes virus classification group and has a large double stranded genome of 240 kilobases. At the immediate early (IE) time after infection, 88% of the viral RNA present is derived from a single region of the genome. Of these immediate early genes at least one was proposed to be a promoter with the capability of regulating the expression of other viral genes.

From this gene region the human CMV promoter was isolated (US patent no. 5,168,062). The promoter contains the common TATA box and CAAT box regulatory regions and the active promoter is thought to extend for 465 bases, a region incorporating a series of nucleotide repeats essential for promoter activity. A total of 767 bases are utilised in the Invitrogen plasmid. This includes the promoter sequence and an additional 295 bases with unknown function.

4.1.1 Assembling the Vector - Summary

The EGFP gene was excised from the pEGFP-1 vector (Clontech, refer to section 3.1.2) and inserted into complementary restriction sites in the multi-cloning site of the pcDNA1amp vector (Invitrogen). This produced a construct from which constitutive EGFP expression could be achieved.

4.2 Results

The EGFP gene was cut from the pEGFP-1 plasmid by a double restriction digest with the enzymes Hind III and Not I (Table A4.2.1). 1μg of plasmid DNA was digested with 10 units of each enzyme in a final volume of 50μl for 3 hours at 37°C. The restriction reaction was run on a 0.8% agarose gel and the 0.8kb fragment corresponding to the EGFP gene was removed and purified from the gel (section 2.5.3.1).

Concomitantly 1μg of the pcDNA1amp (Invitrogen) vector was also digested with Hind III and Not I in the same manner and the major 4.7kb fragment was extracted from an agarose gel as described in section 2.5.3.1.

The pcDNA1amp vector backbone was dephosphorylated with calf intestinal alkaline phosphatase (section 2.5.5), the vector and insert were then ligated overnight at 4°C with T4 DNA ligase (section2.5.6).

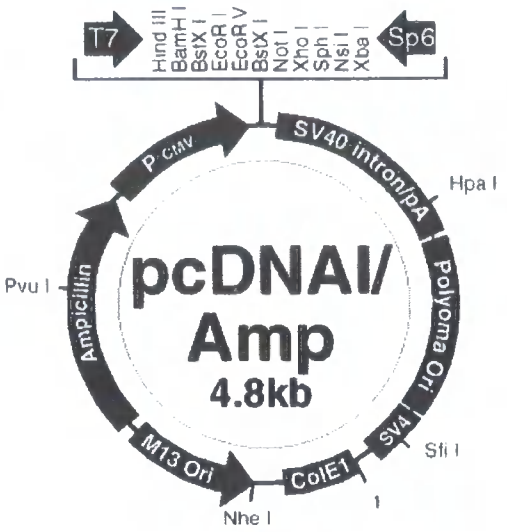
Plasmid	Enzyme	Restriction Site	Fragment Length
pEGFP-1	Hind III and Not I	41 and 820	779, 3421
pcDNA1amp	Hind III and Not I	2964 and 3039	75, 4726

Table A4.2.1. Restriction of the two plasmids for the production of the pCMV-EGFP vector.

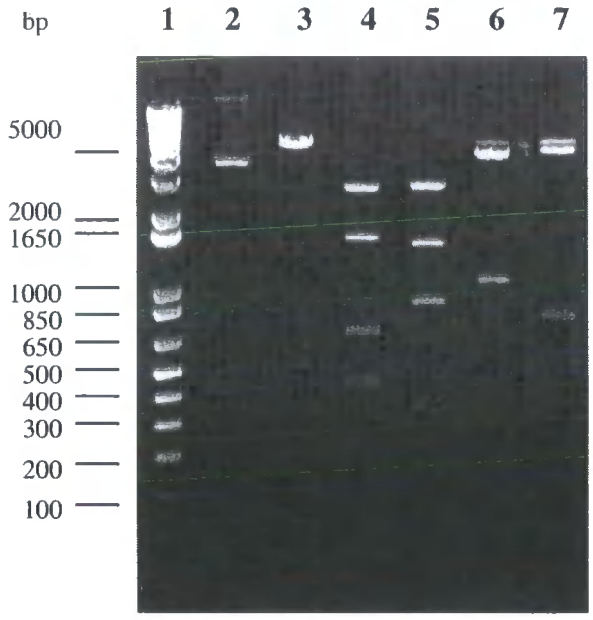
Following calcium chloride transformation (section 2.5.8.1) three colonies were identified, DNA was extracted via the miniprep method (section 2.2.1) and restriction digest (section 2.5.2) identified two that had incorporated the insert (Table A4.2.2 and Figure 4.2.3).

Plasmid	Enzyme	Restriction Site	Fragment Length
pCMV-EGFP	Nde I		linearise 5.6kb
	Hind III and Not I	2964 and 3743	**779**, 4821
	Dra I	1584, 2276, 3940, 4377	692, 1664, 437, 2807
	Dra I and Not I	1584, 2276, 3743, 3940, 4377	692, **1467**, 197, 437, 2807

Table A4.2.2 Restriction map of the pCMV-EGFP plasmid. Fragments highlighted in bold represent the excised EGFP fragment (779bp) and the CMV-EGFP sequence (1467bp).



a.



b.

Figure A4.2.3 The pCMV-EGFP Positive Control Palsmid
a. The EGFP gene was inserted into the Hind III and Not I digested pcDNA1/Amp plasmid (Invitrogen). **b.** Restriction digest of the resulting plasmid pCMV-EGFP. Lane 1 molecular weight markers. Lane 2 uncut CMV-EGFP plasmid DNA. Lane 3-7 pCMV-EGFP DNA restricted with Bam HI, Dra I, Nco I, Pst I and double digest with Hind III and Not I respectively. Lane 7 represents the removal of the inserted EGFP gene (779bp.)

A4.3 Functional Study of Activity of pCMV-EGFP

Once the construct had been prepared, its ability to drive EGFP protein expression in a ubiquitous fashion was assessed. Confluent cultures of COS-7 green monkey kidney cells were transfected with the purified plasmid as described in section 3.3.1.4.

As seen in Figure 3.3.3 successful expression of the protein was demonstrated in a full range of cell types.

Relevant Patent:

US Patent and Trademark Office. Inventor Stinski, M.F. 1990 no. 5,168,062 Transfer vectors and microorganisms containing the human cytomegalovirus immediate early promoter regulatory DNA sequence.

Appendix B.

The Generation of an Alternative Mouse Model. The MBP-EGFP Mouse

Appendix B: Generation of an Alternative Mouse Model. The MBP-EGFP Transgenic Mouse

B1 Introduction

Like the peripheral nervous system, many neurons in the brain require a myelin sheath to enable them to function properly. In the central nervous system the task of producing the myelin sheath is performed by the oligodendrocyte. These cells are found in the white matter of the brain, where individual cells extend processes and myelinate many nerve fibres.

B1.1 Development of the Oligodendrocyte Lineage

Much of the work investigating the development of the oligodendrocyte has been carried out in the rat optic nerve as this region of the CNS is relatively simple. It does not contain any neuronal cell bodies and has only three types of glial cell, type I and II astrocytes, and oliogdendrocytes. Studies on optic nerve cultures have shown that two distinct lineages are involved in the formation of the glial populations in the optic nerve; oligodendrocytes and type II astrocytes share a common precursor while type I astrocytes develop from a separate cell type (Raff *et al.*, 1983; 1984). The common lineage of oligodendrocytes and type II astrocytes may relate to function, in that both are found to contribute structurally to the node of Ranvier (ffrench-Constant and Raff, 1986).

The common precursor cell, the pre-O2A, is derived from the subventricular zone of the forebrain and the ventral spinal cord (Levine and Goldman, 1988a,b). These small, round, glossy cells are distinguished antigenetically from later cell types as they are negative for the A2B5 antibody that binds to polysialoganglioside proteins (Eisenbarth *et al.*, 1979; Gard and Pfieffer, 1990; Hardy and Reynolds, 1993, Asou *et al.*, 1995).

The precursor cells mature to become O2A progenitors; process bearing cells that are positive for the A2B5 antigen. These latter cells are highly proliferative and can

differentiate into either oligodendrocytes or type II astrocytes (Levi *et al.*, 1986). Both of the final cell types divide infrequently, if at all, with the main expansion of the population arising from the initial proliferation of the progenitor cells (Skoff *et al.*, 1976; Miller *et al.*, 1985). In the rat, oligodendrocyes first begin to appear at the day of birth, and the O2A progenitor cells continue to divide and differentiate for at least the next two weeks (Skoff *et al.*, 1976). Type II astrocytes are thought to begin developing from the same progenitors at later stages (Miller *et al.*, 1985). Experiments suggest that in the absence of signals from any other cell type the oligodendrocyte is the default pathway of differentiation for the O2A progenitor cell (Temple and Raff, 1985).

In vivo the O2A progenitor cells continue to proliferate over a period of weeks. *In vitro,* proliferation stops and differentiation begins within two days. The outcome of the differentiation is determined by the environmental conditions, cells that are maintained in 10% foetal calf serum (FCS) differentiate into type II astrocytes; in its absence oligodendrocytes are formed (Raff *et al.*, 1983; Raff *et al.*, 1984). The correct timing of differentiation *in vitro* can be restored by culturing oligodendrocyte precursors on a monolayer of type I astrocytes (Noble and Murray, 1984; Raff *et al.*, 1985). The mitogenic factor released by these cells was found to be platelet derived growth factor (PDGF), which itself can be used to reset the timing mechanism (Noble *et al.*, 1988; Richardson *et al.*, 1988; Raff *et al.*, 1988).

Experiments with the daughter cells of a single precursor suggest that in addition to regulation by PDGF, an intrinsic timing mechanism occurs within the cell. When stimulated by PDGF to divide two isolated daughter cells from a common precursor will do so the same number of times before committing to differentiation (Temple and Raff, 1986). Analysis of the proliferation rates at different temperatures also points to an underlying mechanism. Oligodendrocyte precursors cultured at 33°C divide slower than those at 37°C. However they stop dividing and differentiate after fewer divisions, so the cells are not "counting" the number of divisions but are measuring time in some other way (Gao *et al.*, 1997).

While it has been demonstrated that the timing mechanism requires the presence of PDGF, in the absence of other factors it is not sufficient in itself to completely restore

the pattern of *in vivo* development. Precursor cells cultured in a medium that contains PDGF but lacking other elements, will continue to proliferate without subsequent differentiation. If either thyroid hormone or retinoic acid is then added to the media, differentiation will begin (Barres *et al.*, 1994).

It is likely that any timing mechanism that controls the proliferation of cells and their division must in some way involve the cell cycle. The cell cycle is controlled by many factors including the cyclin dependant kinases (cdk), these are activated to regulate the different phases of the cell cycle at the correct time and in the right sequence (Morgan, 1995; Lees, 1995). In turn they are influenced by cdk inhibitors, one of these, p27 has been associated with oligodendrocyte development. Levels of p27 accumulate within oligodendrocyte precursors with successive divisions, a threshold level may be the trigger for differentiation (Durand *et al.*, 1997). Mice deficient in p27 have precursors that divide within the normal cell cycle time, but go through more divisions before differentiation than wild type (Durand *et al.*, 1998).

As differentiation proceeds the immature oligodendrocytes gain a surface molecule recognised by the O4 antigen. This precedes other markers such as the oligodendrocyte specific cell surface galactocerebroside (GalC) , (Raff *et al.*, 1978). GalC expression is first seen at birth, subsequently A2B5 staining is lost while O4 antigenicity remains (Levi *et al.*, 1987; Curtis *et al.*, 1988).

B1.2 The Pattern of Myelin Protein Expression in the CNS

As the oligodendrocyte begins to produce long processes these identify an appropriate axon and begin to ensheath it, wrapping layers of membrane spirally around the axon to form the internode. Each oligodendrocyte can form up to 60 internodes on a number of different axons (Matthews and Duncan, 1971). In contrast to the Schwann cells, oligodendrocytes do not rely absolutely on axonal contact and regulation to produce compact myelin. *In vitro* studies have shown that oligodendrocytes can accumulate myelin proteins in the absence of axons (Dubois-Dalqc *et al.*, 1986) and these can be

actively assembled into structures resembling myelin (Poduslo *et al.*, 1982; Bradel and Prince, 1983; Sarlieve, *et al.*, 1983).

The composition of myelin protein is also different from that in the PNS. Proteolipid protein (PLP), which accounts for approximately 50% of all CNS myelin protein is not found in the PNS. While the major structural protein of the PNS myelin, protein zero (P_O) is absent from the CNS. In addition, the transmembrane spanning protein PMP-22 is restricted to the PNS. The CNS and PNS however, both possess the myelin basic protein (MBP) family of proteins and the transmembrane myelin associated glycoprotein (MAG).

In vitro studies have elucidated the pattern of myelin protein expression in the CNS. Oligodendrocytes are first seen at birth and are identified by expression of GalC (Raff *et al.*, 1978). After five days myelin basic proteins are found in the brain (Roussel and Nussbaum, 1981). In cultured oligodendroctes, MBP is found in the cytoplasm of the oligodendrocyte 5 days after plating and within their processes by 10-15 days (Dubois-Dalcq *et al.*, 1986).

The initial expression of MAG closely follows that of MBP, however it is present in the oligodendrocyte projections 9 days after plating. The expression of PLP begins later than that of MBP and MAG. It is seen first at 7 to 9 days in the cell body, and at 14 to 15 days in the oligodendrocyte processes (Dubois-Dalcq, 1986). A similar delay in PLP expression in comparison to MBP is found *in vivo* (Campagnoni and Hunkeler, 1980).

B1.3 Myelin Basic Protein

Myelin basic protein is shown to be one of the first myelin specific proteins to be expressed in both Schwann cells and oligodendrocytes as the myelin sheath begins to be constructed. MBP constitutes 30% of the total protein isolated from CNS myelin. The myelin basic protein family comprises of several proteins ranging from 14 to 21.5kDa that are formed by the alternative splicing of the MBP gene (de Ferra *et al.*, 1985; Takahashi *et al.*, 1985; Newman *et al.*, 1987). In the mouse and rat the major isoforms 21.5kDa, 18.5kDa, 17kDa and 14kDa are present in the relative amounts of 1:10:3.5:35

(Barbarese *et al.*, 1978). Antigenically they are all identifiable with an antibody raised against the smallest, 14kDa MBP isoform (Fritz and Chou, 1983).

MBP proteins are synthesised on free polysomes in the cytoplasm (Campagnoni *et al.*, 1978, 1980; Colman *et al.*, 1983) and following the development of the oligodendroglial processes are translocated to the plasma membrane (Verity and Campagnoni, 1988; Amur Umarjee *et al.*, 1990). Once located at the site of myelin compaction MBP is intimately involved in maintaining the structure of the sheath.

B1.3.1 The MBP gene is part of a complex gene organisation

The classical *MBP* gene in mouse contains seven exons stretched across 32kb of chromosome 18. Alternative splicing of these exons is responsible for the range of different MBP isoforms found (de Ferra *et al.*, 1985; Roach *et al.*, 1985). However, a number of MBP immunoreactive transcripts exist in both rodent brain and glial cells that have not been identified as MBP isoforms (Barbareses and Pfieffer, 1981; Barbareses *et al.*, 1983; Carson *et al.*, 1983).

Kitamura *et al.* (1990) isolated M41-MBP mRNA, believed to be derived from the MBP transcription start site. It codes for a 14kDa MBP protein, however, it has an extended 5' region in comparison to other MBP mRNA species. A number of other, similar clones were recognized and analysis of these have identified further exons that described a new gene (Campagnoni *et al.*, 1993). The new region was designated *Golli-mbp* as the gene products are expressed in the *oli*godendrocyte *li*neage in the brain and encompass the *MBP* gene. It spans 105 kb of chromosome 18 encompassing 11 exons, and describes two overlapping transcriptional units. The golli-mbp exons 5A and 5B that are at the beginning of the MBP transcript also combine with exon 5C to form the end of the BG21 gene. The complex exon 5 region comprises three distinct regions that are alternatively spliced and are able to confer promoter activity in their own right.

The multiple products of this gene region are expressed in a temporal manner at different stages of differentiation of the oligodendrocyte lineage. It is thought likely that the *golli-mbp* gene products are involved during the differentiation of the oligodendrocyte prior to the elaboration of the myelin sheath. Subsequently the MBP

proteins are intimately involved in maintaining the mature myelin (Campagnoni *et al.*, 1993).

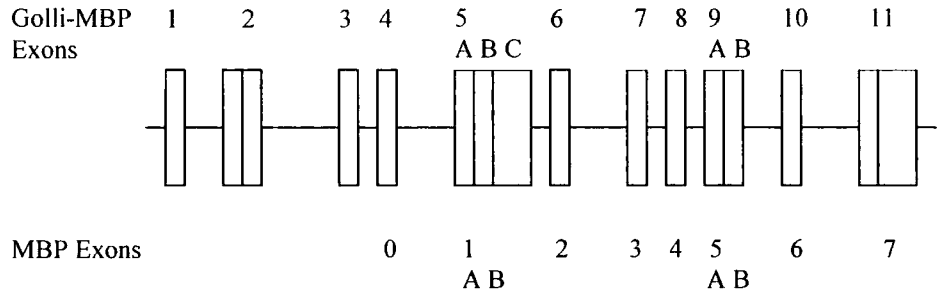


Figure B1.3.1 Representation of the organisation of the Golli-MBP gene region. The classical MBP gene comprises of seven coding exons. This was later found to be part of a larger transcriptional unit the *Golli-mbp* gene. Not to scale. Based on Campagnoni *et al.*, 1993

B1.3.2 The Structure and Function of MBP

Developmental regulation of the expression of the different MBP isoforms, and distinct localisation of individual isoforms suggests diverse function within the family group. The exon II of the MBP gene appears crucial in determining this.

Immunofluorescence localisation of individually expressed transcripts has identified two populations of MBP proteins within the oligodendrocyte. Of the major myelin isoforms studied the 14 and 18.5kDa proteins were found at the plasma membrane whilst the transcripts of the 17 and 21.5kDa isoforms, that include information from exon II were found throughout the cytoplasm and within the nucleus (Staugaitis *et al.*, 1990; Allinquant *et al.*, 1991).

Developmental studies have also identified MBP within the cytoplasm and nucleus of immature oligodendrocytes (Sternberger *et al.*, 1978; Hartman *et al.*, 1979; Roussel and Nussbaum, 1981). These proteins have been confirmed as exon II-containing isoforms. They reach high levels of expression in immature oligodendrocytes and in the early stages of myelination, but as myelination proceeds expression then decreases (Barbarese *et al.*, 1978; Carson *et al.*, 1983; Jordan *et al*, 1989a). The levels of non-exon II-containing transcripts however increase and remain high following myelination.

Jordan *et al.* (1989b) studied the behaviour of the MBP isoforms following nerve injury and found a dramatic increase in the amount of the exon II-containing MBP transcripts at the lesion site. Specifically the MBP isoforms were seen in the perinuclear cytoplasm of the oligodendrocytes. A change in the ratio of exon II and exon I containing transcripts signalled the increase in other MBP transcripts with a more diffuse distribution.

The transport of the exon II-containing transcripts to the nucleus has been shown to be an active process (Pedraza *et al.,* 1997). It is proposed that these early isoforms of MBP are involved in oligodendrocyte maturation, necessary before myelination can occur. Once the oligodendrocyte processes have formed, later MBP isoforms are produced and translocated to the plasma membrane (Verity and Campagnoni, 1988; Amur Umarjee *et al.*, 1990).

In mature oligodendrocytes MBP can be found at the site of the major dense line (MDL), an electron micrographic image that represents the apposition of the cytoplasmic faces of adjacent myelin layers (Omlin *et al.,* 1982). The homozygous *shiverer* mouse produces very little MBP (Kirchner and Ganser,1980; Campagnoni *et al.*, 1984) and fails to produce compact myelin. Electonmicrographs show a loss of the MDL (Privat *et al.,* 1979; Rosenbluth, 1980a). A deletion in the MBP gene is responsible for the *shiverer* mouse phenotype (Kitamura *et al.,* 1985; Roach *et al.*, 1985). This observation suggests that MBP is likely to be integral to the maintenance of the MDL of compact myelin, acting as an intracellular adhesion molecule.

B1.3.4 Transgenic Strategies to Determine the Function of MBP.

A number of natural mouse mutants exist whose phenotype is seen to affect the nervous system. Two of these phenotypes have been shown to be related to mutations in the MBP gene, the *shiverer* mouse *shi,* and the myelin deficient mouse shi^{mld}.

B1.3.4.1 The *Shiverer* Mouse

The *Shiverer* mouse displays a coarse action tremor beginning at the end of the second postnatal week, and progressing with age. Convulsions begin after a few months and become increasingly severe leading to premature death at 90-150 days. *Shiverer* is an autosomal recessive mutation (Biddle *et al.*, 1973; Chernoff *et al.*, 1981) characterised by an almost total lack of CNS myelin (Bird *et al.*, 1978; Rosenbluth 1980a) with all MBP isoforms absent (Depouey *et al*, 1979; Kirschner and Ganser, 1980; Mikoshiba *et al.*, 1981; Barbarese *et al.*, 1983). The loss of the major dense line in the CNS further implicates the MBP gene as the site of this mutation (Privat *et al.*, 1979). However in the peripheral nervous system of this mouse, functional myelin sheaths are present although they are often thinner than in wild type mice (Rosenbluth 1980b; Peterson and Bray, 1984).

Both the *MBP* gene and the *shi* mutation had been mapped to mouse chromosome 18 (Sidman *et al.*, 1985; Roach *et al.*, 1985) and it has been shown that the *MBP* gene in fact is deleted from the second intron to 2kb 3' of the last exon in the *shiverer* mouse (Roach *et al.*, 1985; Takahashi *et al.*, 1985; Molineaux *et al.*, 1986).

Transgenic studies have caused both the induction and the rescue of the *shiverer* phenotype through manipulation of the *MBP* gene. Readhead *et al.* (1987) were able to rescue the mutant phenotype by incorporating the full length sequence of the *MBP* gene into *shiverer* mice. It has been shown that rescue can occur with the expression of only a single MBP isoform. Kimura *et al.* (1989) introduced a minigene encoding the 14kDa MBP isoform, driven by 1.3kb of 5' region into a *shiverer* mouse with restoration of myelin formation that in turn alleviates the tremoring phenotype. They have also shown recently that introducing the minor MBP isoform, the 17.5 kDa protein is also sufficient to rescue the *shiverer* phenotype (Kimura, *et al.*, 1998).

Conversely the *shiverer* phenotype can be induced through the expression of antisense MBP mRNA. Antisense mRNA is thought to bind to the sense mRNA preventing protein formation. This manipulation was found to convert the normal phenotype of heterozygous *shi* mice to a more severe homozygous *shiverer* phenotype. Immunohistochemical analysis of the brains of these mice showed mosaic loss of MBP expression and myelination (Katsuki *et al.*, 1988).

B1.3.4.2 The Myelin Deficient Mouse

The myelin deficient mouse, *shi*^{mld} exhibits a phenotype similar to that of *shi* mice. Tremors begin at about day 12 and continue throughout life. Convulsive seizures begin at 1-2 months and lead to early death at 5-9 months (Doolittle and Schweikart, 1977). The CNS myelin of *shi*^{mld} mice is severley hypomyelinated (Doolittle and Schweikart, 1977; Matthieu *et al.*, 1981). Unlike *shiverer* however, all MBP isoforms are seen in the *shi*^{mld} mouse but at reduced levels and with abnormal developmental expression (Matthieu *et al.*, 1984; Roch *et al.*, 1986; Popko *et al.*, 1987).

The mutation in myelin deficient mice is a complex gene duplication that results in one intact copy of the *MBP* gene, and a second that contains an inversion (Okano *et al.*, 1987; 1988a; Popko *et al.*, 1988). It is proposed that the inverted gene produces antisense mRNA of the MBP code and that this can form duplexes with the sense mRNA from the intact gene. This reduces the amount of MBP mRNA and therefore the protein (Okano *et al.*, 1988b; Tosic *et al.*, 1990).

Transgenic mice have been produced in which the *shi*^{mld} phenotype could be alleviated by introduction of further copies of the intact MBP gene (Popko *et al.*, 1987).

A number of different lengths of the MBP 5' untranslated region (UTR) have been used to drive reporter gene expression in different transgenic mouse lines created to study the expression of MBP (for review see Ikenaka and Kagawa, 1995). The use of *lacZ* as a reporter gene has allowed analysis of the temporal and spatial expression pattern of MBP throughout the CNS (Gow *et al.*, 1992; Foran and Peterson, 1992; Wrabetz *et al.*, 1998). These transgenic animals, however, do not express the reporter gene in the Schwann cells of the PNS. Some regulatory element is lacking that is necessary for the peripheral expression of MBP.

Detailed characterisation of the key regulatory regions within these promoter regions has identified a number of enhancer and repressor *cis* acting elements that affect the expression of MBP.

B1.3.5 Characterisation of the Myelin Basic Protein Promoter.

Kimura *et al.* (1989) were able to show that 1.3kb of the upstream sequence of the MBP gene were able to confer promoter activity sufficient to drive expression of an MBP "minigene". This contained the cDNA sequence for the 14Kda MBP species and was expressed to alleviate the murine *shiverer* phenotype. Initial analysis identified a distal and proximal region within the promoter (-253 to –54 and -53 to +60 respectively) that were capable of tissue specific transcription (Tamura *et al.*, 1989). A core promoter within the proximal region was identified at bases –36 to +12, and the region between -22 and –17 was shown to be important for efficient transcription in brain nuclear extract assays (Tamura *et al.*, 1991, 1990). Additionally an activator sequence designated MB1, –14 to –50 was also found with a 10 base pair motif that increased transcriptional activity (Devine-Beach *et al.*, 1991).

The distal promoter region has been extensively studied. Miura *et al.* (1989) incorporated the 1.3kb promoter region into a plasmid containing the gene for *lacZ,* and carried out a series of deletions within the 5' region. The effects of these deletions were assessed by expression studies in a mouse neuronal/glial hybrid cell line, NG108-15 (Amano *et al.*, 1974) to determine regulatory important regions within it.

Miura *et al.* were able to identify TATA and CAAT box regions at –34 and –85 respectively and a GC rich region at –92bp. This compares with the position of the TATA and CAAT boxes in the rat and human found at –30 and –80 respectively in both species (Wrabetz *et al.*, 1993). In addition the sequence for an NF1 transcription factor binding site was found to be located at –125 (Leegwater *et al.*, 1986) and a sequence sharing homology with the SV40 enhancer core was found at –648 (Weiher *et al.*, 1983). When a large fragment from –1318 to –254 was deleted from the promoter region the β-galactosidase enzyme activity increased dramatically suggesting that within this region elements exist that repress MBP promoter function. Deletion studies also identified the region between bases –208 and –139 as a *cis*-acting enhancer sequence. In addition bases –139 to –118 and –89 to –75 (which includes the CAAT box) were also shown to be required for efficient transcription as their removal lead to reduced promoter activity.

Wrabetz *et al.* (1993), in an investigation of the human MBP promoter, were able to show that as little as 149 bp upstream of the MBP gene were sufficient to drive chloramphenicol transferase (CAT) to a level of expression 8 fold greater than that seen with a promoterless control specifically in oligodendrocytes. Detailed investigation of this 149 base pair length identified both negative and positive *cis*-acting elements within it.

A deletion of base pairs –149 to –108 gave rise to an increase in CAT expression, representing an *in vivo* **negative** effect on the functional activity of the promoter. This includes the –124 to –110 region previously identified as the consensus NF1 site. Aoyama *et al.* (1990) identified the sequence from –130 to –111 in the mouse MBP promoter, also containing the NF-I site, as a **positive** cis-acting element that is necessary for brain specific expression of MBP. The reasons for these conflicting results are unknown but they may be due to the extent of the deletions or the protocols used to assay the expression. The human and murine promoters have been shown to be 90% homologous (Kamholz and Wrabetz, 1992).

The proximal section of the region under investigation, –102 to –95 shows positive functional activity and interrupts the motif GGAGCCG, an element that has also been identified in the PLP promoter (Berndt *et al.*, 1992; Nave and Lemke, 1991; Kim and Hudson, 1990). Deletion of the TATA box region, -41 to –14 reduced CAT expression levels to that of the control.

Wrabetz *et al.* (1993) proposed a mechanism of action for the positive and negative *cis*-acting elements that they identified. They suggested that the positive elements may be required for MBP activation whilst the negative region restricts expression in a cell specific manner, a situation seen with the glycophorin B promoter (Rahuel *et al.*, 1992). This idea is supported by the observation that the original promoter –149 to +81 was only capable of expression in oligodendrocytes. However when the negative acting element was removed the remaining promoter region was able to function in COS-7 cells.

For a summary of the regulatory elements within the MBP promoter see Table C1.3.5

Element	Location (base pairs)	Regulatory Role within MBP Promoter
TATA box	-34 mouse, -30 rat	Deletion from rat (-41 to –14) destroys promoter activity (Wrabetz *et al.*, 1993)
CAAT box	-85 mouse, -80 rat	Necessary for efficient transcription. Deletion reduces promoter activity (Miura *et al.*, 1989)
GC rich box	-92	Deletion of this region has no effect (Miura *et al.*, 1989)
NF1 binding site	-124 to -110	Aoyama et al. (1990) identified as necessary for brain specific expression. Wrabetz et al. (1993) however found located within negative regulatory region –149 to -102
Repressor region	-1318 to –254	Deletion causes increase in lacZ expression, region may contain repressor elments (Miura *et al.*, 1989)
Enhancer region	-208 to -139, -139 to -118	Deletion causes reduced activity: necessary for efficient transcription (Miura *et al.*, 1989)
Negative *cis*-acting element	-149 to -102	Region of negative regulatory element. Can be subdivided into a single negative region at -128 to –116 and two positive from -149 to –128 and –116 to –102 (Wrabetz *et al.*, 1993)
Positive *cis*-acting element	-102 to -95	Deletion reduces activity, therefore contains positive elements. Interupts motif found in PLP promoter (Nave and Lemke, 1991)
MB 1	-50 to -14	Gives cell type specific expression of MBP (Devine-Beach *et al.*, 1991)
MB 3	-130 to -94	Interacts with nuclear proteins from mouse brain at various stages during development (Haque *et al.*, 1995)
Thyroid hormone response Element	-186 to -163	Classical thyroid hormone response element (Farsetti *et al.*, 1992)

TableB1.3.5. Regulatory regions identified within the MBP promoter.

B1.3.5.1 Hormonal Influence on Expression

Thyroid hormone has been shown to be vital for the development of the CNS with a critical active period at 10-15 days after birth. In its absence, retardation occurs and the processes of cellular differentiation within the brain are disrupted (Schwartz, 1983). In animals with hypothyroidism the process of myelination is also severely impaired, and there is a decrease in the final levels of myelin (Balazs *et al.*, 1969). Farsetti *et al.*, (1992) were able to show that in the same animals, despite having a normal timing of expression, the levels of MBP proteins were greatly reduced. In animals from which the thyroid was removed at 5-6 weeks of age the MBP levels subsequently decreased and were rescued by the administration of thyroxine.

In mouse cerebral cell cultures thyroid hormone was found to effect the expression of MBP mRNA (Shanker *et al.*, 1987) and further analysis of the MBP promoter region has identified a thyroid response element within it (Farsetti *et al.*, 1992; 1997). In order to effect its regulation through the thyroid hormone response element, the thyroid hormone first has to form a complex with a thyroid hormone receptor. A number of different isoforms of the thyroid receptor have been found localised to the oligodendrocyte nucleus (Carlson *et al.*, 1994).

B1.4 Project Aims

It can be seen that the MBP promoter region has been extensively studied and that many of its functional components have been determined. The production of the PMP-22 P1-EGFP transgenic mouse was considered a high risk project and to ensure some level of result within the PhD an alternative transgenic mouse was proposed. This would be prepared by an institution that had expertise in the field of microinjection and transgenic production and so increase the probability of success.

To this aim, a construct was prepared in which the proven activity of the MBP promoter was utilised to drive the expression of the EGFP reporter gene. This construct was then to be prepared for microinjection and sent to the Karolinska Institute in Sweden. The resulting transgenic line would be complimentary to the PMP-22 P1-EGFP mouse as it would be predicted to express EGFP in the central nervous system.

B2 Results

B2.1 Optimising the PCR conditions

Primers were designed against the published MBP promoter sequence from the NCBI Genbank data base, accession number M24410 (Miura *et al.*, 1989). The primers were designed in accordance with the guidelines noted in section 3.2.1.

Oligonucleotide	Sequence 5' to 3'	Position in Promoter
Forward	CAG ATC TTA TTC CTC ACC	-1298 to -1281
Reverse	TGG TCC ATG GTA CTT GCT	+105 to +122

Table B2.1.1 Oligonucleotide primers designed to isolate the MBP promoter.

The promoter was extracted from mouse genomic DNA by the polymerase chain reaction. The following reaction was set up in a 0.25ml microcentrifuge tube and maintained on ice.

1x taq DNA polymerase buffer (10mM Tria pH 8.3, $MgCl_2$, 50mM KCl, 0.1mg/ml gelatin)
dNTP's 0.2mM (dATP/ dTTP/ dCTP/ dGTP)
Forward and reverse primers at a concentration of 1μM
$MgCl_2$ 0.5 to 2.5 mM range
100-250ng genomic DNA
Sterile H_2O to a final volume of 100μl

A number of reactions were prepared in order to optimise the interaction between the primers and the DNA at different $MgCl_2$ concentrations.

Each reaction was spun briefly to mix the components. The tubes were placed in a Techne PHC-3 Thermal cycler and the first round of denaturation was performed before the addition of 1.0 unit of Taq DNA polymerase. Cycling parameters were as described below:

		Temp (°C)	Time (seconds)
One cycle:	denaturation	92°C	300
Thirty cycles:	denaturation	92°C	45
	annealing	50°C	45
	extension	72°C	45
One cycle:	extension	72°C	300

Table B2.1.2 Description of the PCR protocol used to isolate the MBP promoter from genomic DNA.

Control reactions were performed at the same time to ensure there was no contamination with unwanted DNA. These consisted of reactions with either no DNA, no enzyme or no primers.

B2.2 Identification of the reaction products.

An aliquot of each PCR reaction product was separated on a 1.2% agarose gel by electrophoresis. A reaction product at the predicted molecular weight of 1.4kb was found. Restriction digest of this product suggested that the MBP promoter region had been isolated (see Table B2.2.1 and Figure B2.2.2).

Enzyme	Restriction Site	Fragment Length
Ava I	555, 571, 1232, 1242	555, 16, 661, 10, 168
Bsp 120I	963	963, 447
Dra I	404, 741	404, 337, 669
Dpn I	2, 142, 544, 1098, 1218, 1374	2, 140, 554, 120, 156 36
Hinf I	593, 1141, 1282	593, 548, 141, 128
Pst I	641	641, 769
Pvu II	1190	1190, 220
Sma I	1242	1242, 168

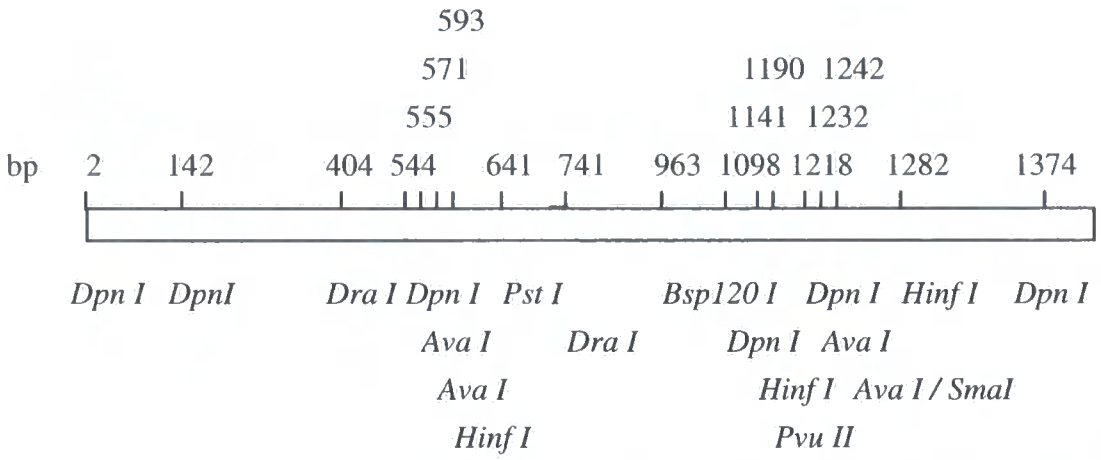
Table B2.2.1 Restriction sites within the MBP promoter. This table shows the restriction sites within the MBP promoter of a number of common enzymes, and the restriction fragment lengths that result from this.

B2.3 Construction of an Intermediate Plasmid

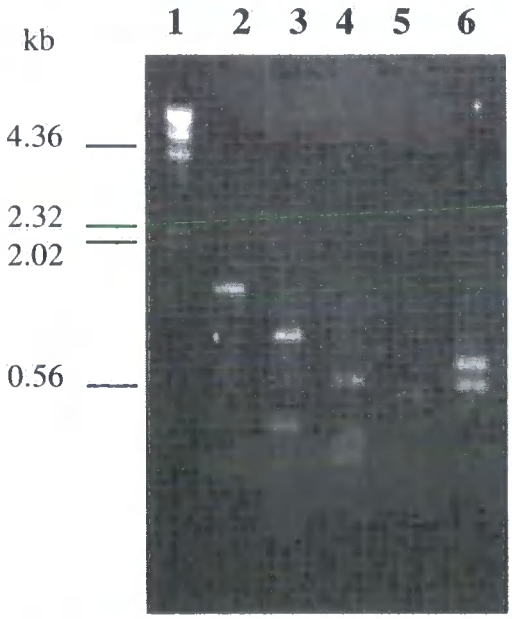
An aliquot of the PCR reaction was run in a low melting point agarose gel of 1.2% and the 1.4kb band was excised. The DNA was isolated from this using the protocol described in section 2.5.3.1.

The purified DNA of the PCR fragment was then ligated into the pGEM T-easy vector (Promega) at 4°C overnight as described in section 2.5.6. Following electro-transformation into XLI-Blue competent cells (section 2.5.8.2) positive colonies were identified by blue white screening (section 2.5.9).

Ten of the potential positive colonies were streaked out and fragments of the colony were incorporated into a PCR reaction as the source of DNA. The PCR strategy devised to isolate the MBP promoter was utilised to identify the positive colonies (Table B2.1.2).



a.



b.

Figure B2.2.2 The Putative MBP Promoter
a. Schematic demonstrating the restriction sites within the MBP promoter sequence. **b.** Restriction digest of the isolated 1.4 kb PCR product. Lane 1, lambda DNA digested with Hind III, lane 2 uncut fragment, lanes 3-6 PCR fragment digested with Bsp 120I, Dra I, Hinf I and Pst I respectively.

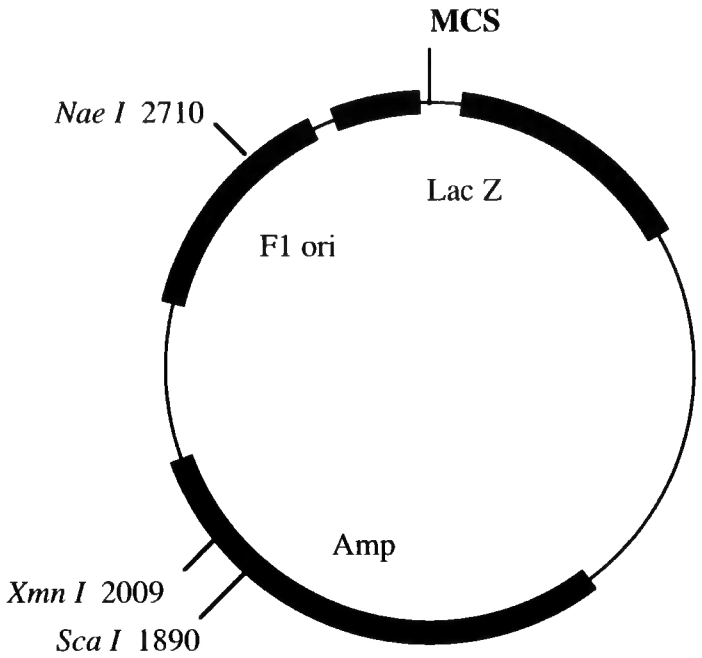
Of the ten initial colonies screened one was found to have successfully ligated the MBP promoter region into the vector backbone.

5ml overnight LB cultures of the positive colony were grown and the plasmid DNA recovered by the miniprep method (section 2.2.1). Restriction digest of this gave corroboration that a positive insertion had occurred (Table B2.3.1and Figure B2.3.2).

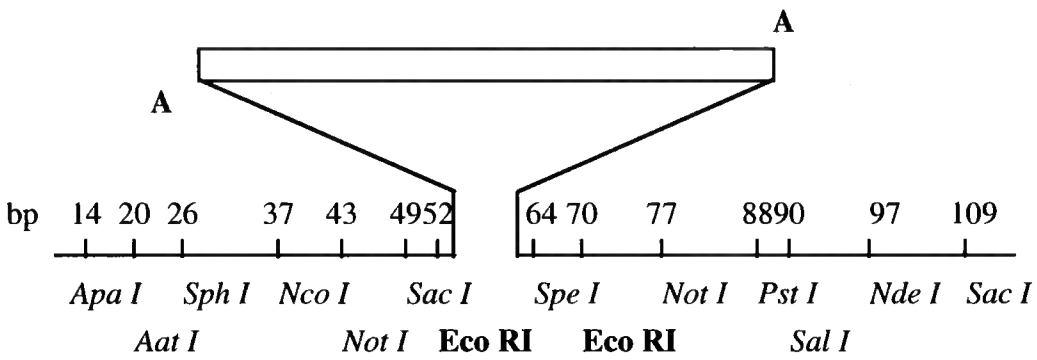
DNA sequencing results from the universal primers T7 and SP6 also confirmed the identity of the insert as that of the MBP promoter.

Enzyme	Restriction Site	Fragment Length
Forward Insertion		
Bsp 120I	10, 1025	1015, 3413
Dra I	466, 803, 2686, 2705, 3397	337, 1883, 19, 692, 1497
Eco RI	52, 1480	1428, 3000
Pst I	703, 1498	795, 3633
Sma I	1304	linearise 4428
Reverse Insertion		
Bsp 120I	10, 509	499, 3929
Dra I	731, 1068, 2686, 2705, 3397	337, 1618, 19, 692, 1762
Eco RI	52, 1480	1428, 3000
Pst I	831, 1498	667, 3761
Sma I	230	linearise 4428

Table B2.3.1. Determination of the Intermediate Plasmid. The MBP promoter-pGEM intermediate plasmid was restricted to ensure the correct insertion of the MBP promoter PCR fragment. This table shows the restriction sites and fragment lengths following the successful integration of the PCR product.

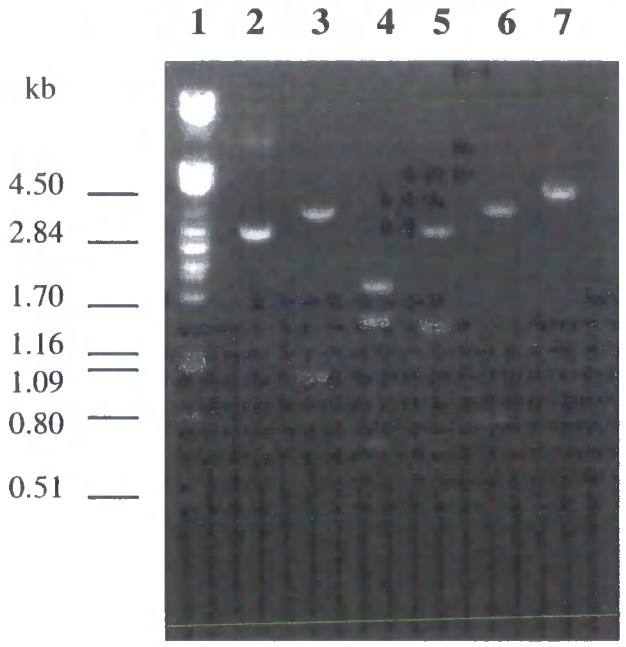


a.



b.

Figure B2.3.2 Intermediate plasmid.
a. Promega pGEM-T easy vector 3kb. **b.** The MBP PCR product, 1.3kb was ligated into the insertion site. The fragment is later easily removed by digestion with flanking enzymes from within the multi-cloning site (MCS).



c.

Figure B2.3.2c Restriction Digest of MBP-pGEM
Restriction digest of the intermediate MBP-pGEM vector in the forward orientation Lane 1, lambda DNA digested with Pst I, Lane 2 uncut vector. Lane 3-7, plasmid digested with Bsp 120I, Dra I, Eco RI, Pst I and Sma I respectively. Eco RI digestion releases the 1.4kb MBP promoter insert.

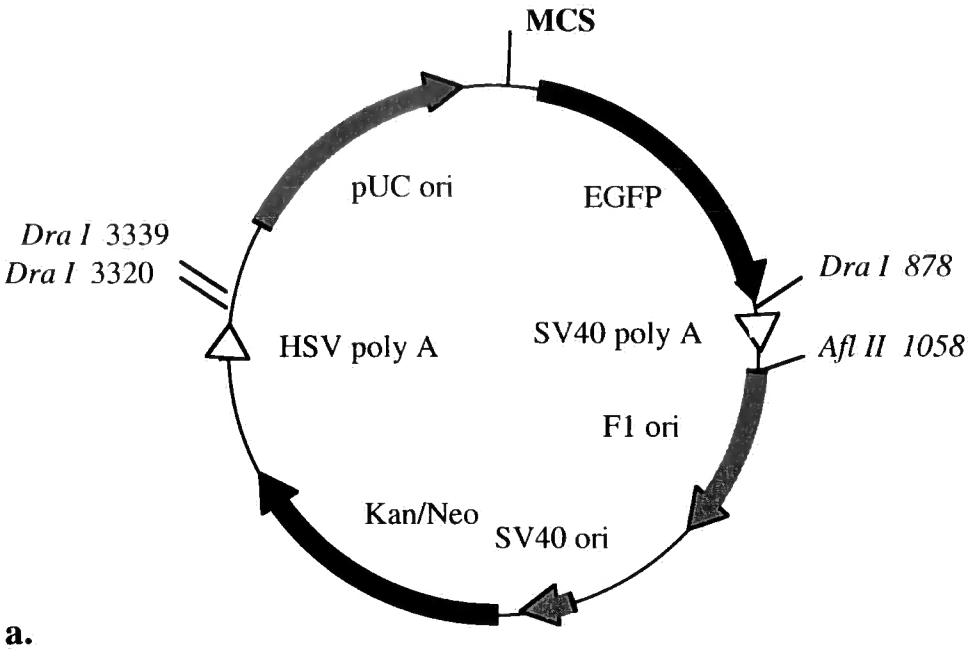
B2.4 Producing the Transgenic Construct

The promoterless pEGFP-1 vector (Clontech) was linearised with the restriction enzyme Eco RI. At the same time the MBP promoter-pGEM T-easy vector was also digested with Eco RI to release the promoter region. The DNA entities were recovered following agarose gel electrophoresis and extraction via the protocol described in section 2.5.3.1. Following dephosphorylation of the vector backbone with calf intestinal alkaline phosphatase enzyme (section 2.5.5) the compatible termini of the vector and insert allowed cohesive ligation as described in section 2.5.6. Following electro-transformation positive colonies were identified by PCR (as described in section B2.1.2) for the presence of the MBP promoter. Of ten initial colonies investigated eight had successfully incorporated the promoter region.

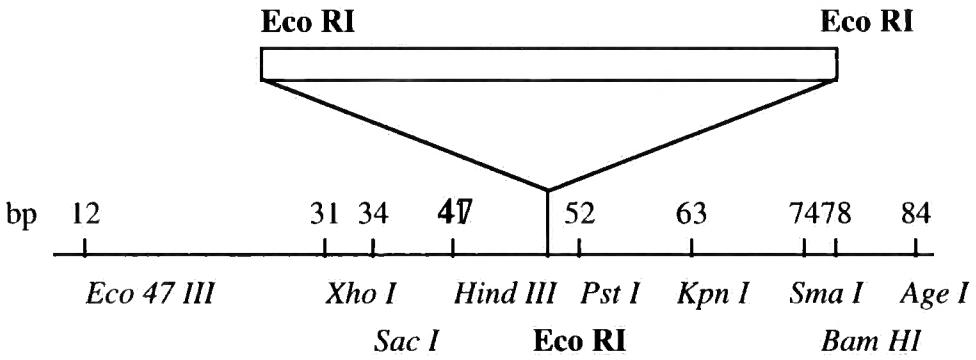
DNA was extracted from 5ml overnight cultures of these colonies and the DNA extracted by the miniprep method (section 2.2.1). Restriction digest identified two clones in which the insert had been ligated in the reverse orientation and six in which it had inserted in the forward direction (Table B2.4.1and Figure B2.4.2).

Enzyme	Restriction Site	Fragment Length
Forward Insertion		
Bbs I	1368	linearise 5466
Bsp 120I	1021, 1382	361, 5105
Dra I	462, 799, 2188, 4630, 4649	337, 1389, 2442, 19, 1279
Pst I	699	linearise 5466
Sal I	Site removed	uncut plasmid DNA
Hind III / Bam HI	41, 1389	1348, 4118
Hind III / Afl II	41, 2368	2327, 3139
Reverse Insertion		
Bbs I	158	linearise 5466
Bsp 120I	505, 1382	877, 4589
Dra I	1064, 727, 2188, 4630 4649	337, 1124, 2442, 19, 1544
Pst I	827	linearise 5466
Sal I	Site removed	uncut plasmid DNA
Hind III / Bam HI	41, 1389	1348, 4118
Hind III / Afl II	41, 2368	2327, 3139

Table B2.4.1. Restriction table of the pMBP-EGFP construct. The orientation of insertion of the MBP promoter into the pEGFP-1 vector backbone was determined by restriction digest. The restriction sites of a number of enzymes and the resulting fragment lengths are shown for either direction of insertion.

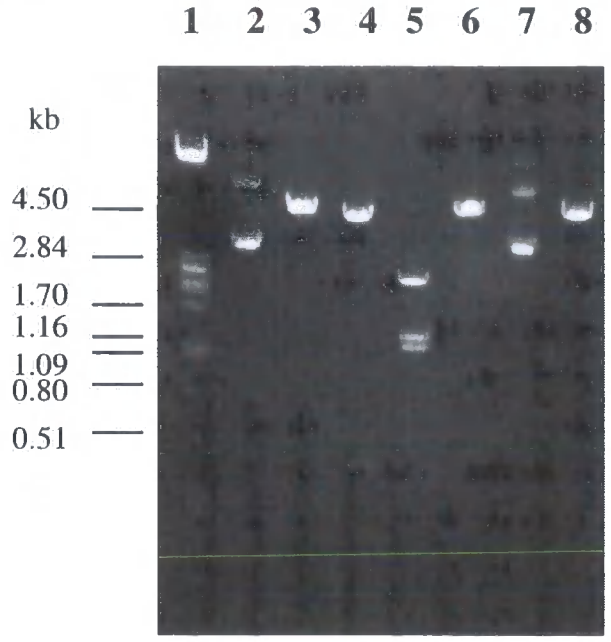


a.



b.

Figure B2.4.2 Production of the final construct.
a. Plasmid map of the Clontech pEGFP-1 vector 4.2kb. Multi-cloningsite (MCS). **b**. Expansion of the MCS showing the insertion of the 1.3kb MBP promoter into the Eco RI restriction site. The EGFP gene is found between base pairs (bp) 97 to 816 of the pEGFP-1 vector



c.

Figure B2.4.2c The final MBP-EGFP construct
Restriction digest of the MBP-EGFP construct. Lane 1, lambda DNA restricted with Pst I. Lane 2 uncut plasmid DNA, lane 3-8 plasmid digested with Bbs I, Bsp 120I, Dra I ,Pst I, Sal and Sma I respectively.

B2.5 Subsequent Manipulation of the MBP Promoter

The reverse primer for the MBP promoter extends into the protein coding region of the MBP gene and so includes the MBP translational start site. In addition the promoterless vector was purposely constructed to possess the necessary translational regulatory elements desired in a plasmid designed to investigate promoter activity. This meant that at this time the construct contained two transcriptional start sites.

In the normal genetic arrangement, a transcriptional start site indicating the beginning of the RNA sequencing region follows a 5' promoter region containing regulatory regions such as a TATA box and CAAT box and transcription factor binding sites. This is then followed by a translational start site, which is positioned in correct alignment with Kozac sequences that aid protein translation. Two translational start sites would render the construct useless, as no functional protein is likely to be derived from it.

A strategy was developed to excise the translational start site from the MBP promoter sequence. Regulatory elements within the MBP promoter were identified to ensure they were not inadvertently removed. In addition the organisation of the endogenous MBP gene was studied. Here the transcriptional and translational start site are located within 47 bases of each other (Kamholz *et al.*, 1988), as the translational start site is removed from the MBP region within the construct the two regions are brought to within 50 base pairs of each other.

The strategy relied upon identifying two enzymes on either side of the site to be removed that only cut the vector once. A Bbs I site at the end of the MBP promoter region (bp1367) and a Sal I restriction site located within the plasmid MCS (1497) both fulfill this criteria.

Subsequently MBP-EGFP vector DNA was digested in a compatible restriction buffer with first Bbs I and then Sal I. To enable re-circularisation the protruding termini were filled in using Klenow enzyme as described in section 2.5.4. The DNA was purified from an agarose gel following the procedures in section 2.5.3.1. A blunt ended ligation was then carried out (section 2.5.6) and the reaction transformed into XLI Blue competent cells (section 2.5.8). A positive colony was identified as previously and confirmed by restriction digest (Table B2.1.2).

B2.6 Functional Studies of the Transgene

To ensure the construct was functional, that it could be expected to express *in vivo,* a number of transfection experiments were carried out. Previous studies have already shown that 1.4 kb of 5' sequence is sufficient to drive oligodendrocyte expression of reporter genes (Kimura *et al.*, 1989; Miura *et al.*, 1989). Our studies were aimed at determining that the regulatory regions necessary for transcription and translation were in context and functional.

The construct was transfected into primary Schwann cells isolated from DRG co-cultures as described previously (section 3.3.2). In addition positive and negative control constructs were also introduced into the cells (section 3.3). Expression was also investigated in a control cell type the green monkey kidney cell line, COS-7 (see section 3.3.2).

B2.6.1 Results of Transfection Study

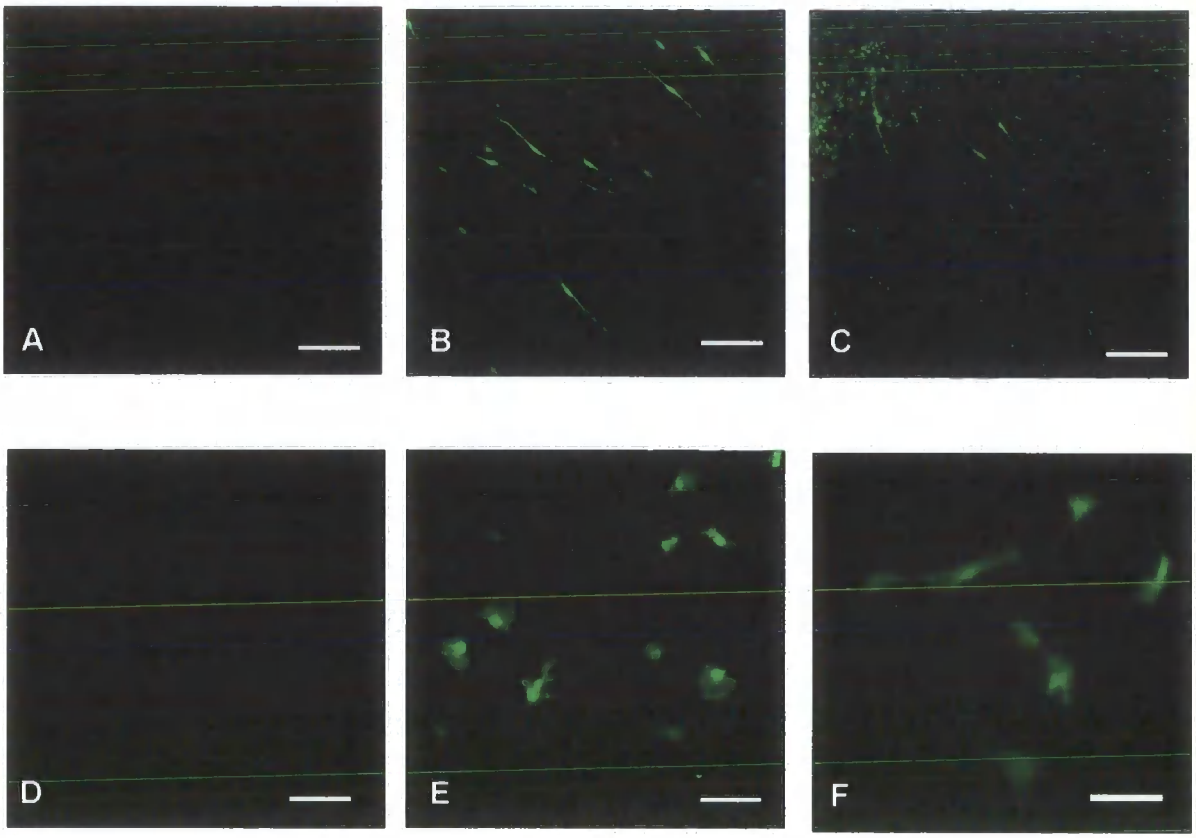
Both cell types successfully expressed EGFP following transfection with the pCMV-EGFP positive control DNA (see appendix A4).

Schwann cells clearly showed expression of EGFP following transfection with the pMBP-EGFP construct (Figure B2.6.1). EGFP fluorescence was seen strongly in the Schwann cell body, with the limits of the Schwann cell processes also visible. Fewer cells were expressing the construct than displayed fluorescence with the constitutively expressing construct.

In addition the control cell line, the green monkey kidney COS-7 cells also strongly expressed the pMBP-EGFP construct. In neither cell type was fluorescence seen with the promoterless EGFP plasmid control.

Figure B2.6.1 Functional studies of the pMBP-EGFP construct.

A-C Transfection of primary Schwann cell co-cultures with the negative control DNA, the positive control pCMV-EGFP plasmid and pMBP-EGFP plasmid respectively. **D-F** Expression of these constructs in COS-7 cells, negative control, positive control and pMBP-EGFP plasmid respectively. Scale bar 100μm


A
B
C
D
E
F

B3 Production of a Transgenic Mouse expressing MBP-EGFP

B3.1 Introduction

The production of a transgenic mouse line in which the MBP promoter was used to drive EGFP expression was conceived as a contingency plan to run parallel to the primary aim of producing the PMP-22 P1-EGFP mouse. For this reason the MBP-EGFP construct was produced at the University of Durham and the DNA prepared for microinjection (section 4.1.1.1). It was then sent to the Karolinska Institute in Sweden where it would be microinjected into fertilised embryos in the same manner as that described in Chapter 4. Tail biopsies from the subsequent pups were then to be sent to Durham for analysis and identification of transgenic pups

B3.2 Microinjection

The pMBP-EGFP plasmid was digested with the restriction enzymes Hind III and Afl II this excised the 2.3kb fragment containing the MBP promoter, EGFP gene and SV40 poly-A tail from the vector. The DNA was purified for microinjection as described in section 4.1.1.1.

In the initial round of microinjection 50 pups resulted from 103 re-implantations. DNA was extracted from 2-4mm tail biopsies as described in section 2.3.2. 5μg of DNA from each mouse was bound to nitrocellulose filter and screened with a ^{32}PdCTP radiolabelled probe to the microinjected fragment (section 2.8). Potential founders were identified and PCR reactions with primers against the MBP-EGFP construct were used to confirm these (Table B3.2.1 and 3.2.2). The PCR reaction failed to identify any positive, potential founder mice.

Oligonucleotide	Sequence	Position in plasmid
Forward MBP	CAG ATC TTA TTC CTC ACC	57 – 74
Reverse TG-2	TTG TAC AGC TCG TCC ATG	2109– 2126

Table B3.2.1 The sequence of primers used to isolate the MBP-EGFP transgene. The forward promoter binds within the MBP promoter region. The reverse primer corresponds to the sequence for the EGFP gene.

These conflicting results necessitated repeated analysis. Genomic DNA was extracted from a second section of tail for each mouse. 5μg of genomic DNA was digested with the restriction enzyme Bam HI. The samples were then run as a Southern blot (section 2.8). The nitrocellulose filter was hybridised with a radiolabelled probe of the full MBP-EGFP sequence (approximately 2.3kb, see section 2.8). The filter was then placed against autoradiograph film, negative results were obtained with all mice.

A second aliquot of DNA was then sent to the Swedish institute for subsequent microinjection. From 5 further rounds of microinjection and re-implantation 43 pups were born and again DNA was extracted from tail biopsies as described previously (section 2.3.2).

Analysis to detect the presence of the transgene began with PCR, using primers against the EGFP gene (see section 5.1). This identified a single mouse, designated 845-5, as a potential founder animal. This was confirmed by PCR against the full length construct MBP-EGFP (see Table B3.2.2). Digestion of the PCR product confirmed the fragment as the inserted MBP-EGFP construct (see Table B3.2.3 and Figure B3.2.4).

		Temp (°C)	Time (seconds)
Two cycles:	denaturation	92	300
	annealing	60	45
	extension	72	45
Two cycles:	denaturation	92	45
	annealing	59	45
	extension	72	45

the annealing temperature was reduced by a further degree every second cycle until the optimum annealing temperature of 50°C was reached

Fifteen cycles:	denaturing	92	45
	annealing	50	45
	extension	72	45
One cycle:	extension	72	300

Table B3.2.2 PCR protocol used to isolate the MBP-EGFP transgene from genomic DNA. The touchdown PCR method was used to isolate the MBP-EGFP transgene from genomic DNA of potential founder animals. Annealing was begun at 68°C and subsequently reduced during the following cycles to the optimum annealing temperature of 58°C. PCR.

Enzyme	Restriction Site	Fragment Length
Bam HI	1335	1335, 735
Bsp 120I	963, 1328	963, 365, 742
Dra I	404, 741	404, 337, 1329
Pvu II	1190	1190, 880

Table B3.2.3 Restriction sites found within the MBP-EGFP promoter used to identify PCR reaction products from potential founders.

The result was further validated by Southern blot of 845-5 (section 2.8). 5μg of the genomic DNA was digested with the restriction enzyme Bam HI and ran in an agarose gel. The gel was then blotted to nitrocellulose filter that was subsequently hybridised with a ^{32}PdCTP labeled probe of MBP-EGFP. Autoradiographs showed the incorporation of the transgene in the genomic DNA of this mouse (Figure B3.2.5).

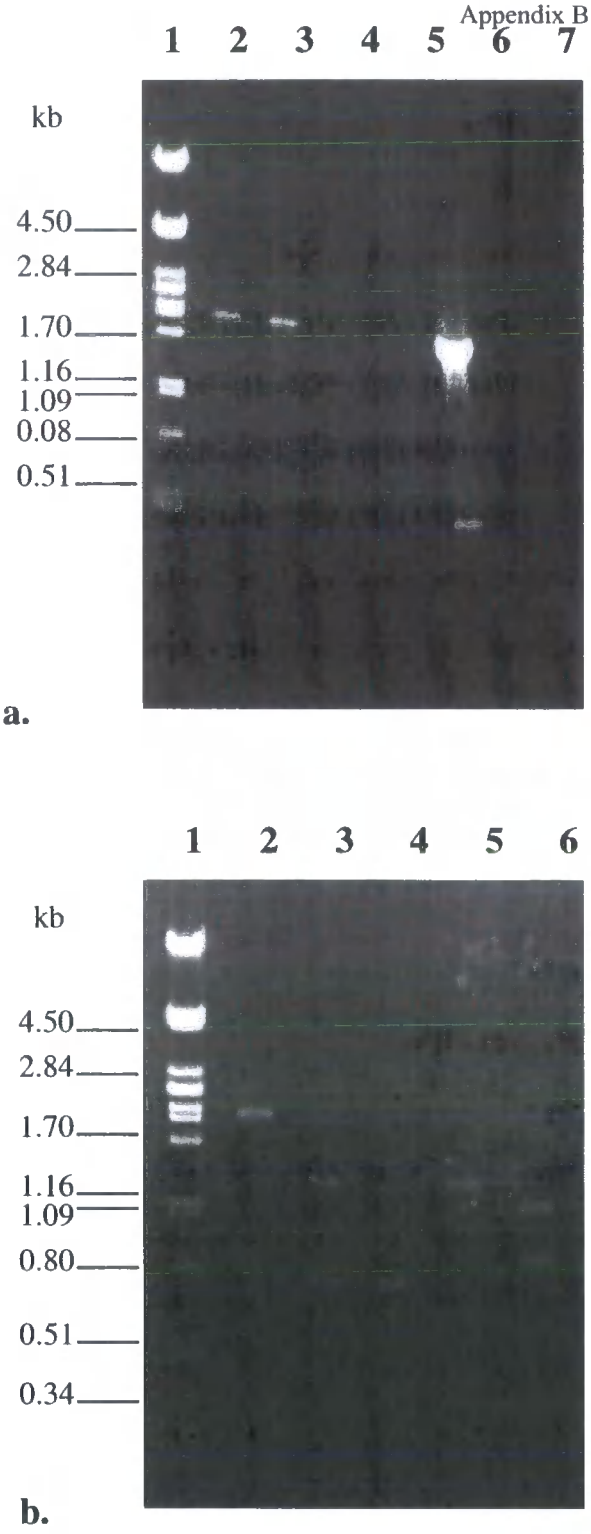


Figure B3.2.4 Identification of Transgenic Mice.
Genomic DNA from the four male offspring of founder 845-5 was screened for the presence of the MBP-EGFP transgene. **a.** Lane 1 molecular weight markers. Lane 2 845-5 founder. Lanes 3-6 F1 pups 845-5/1 to 845-5/4 respectively. Lane 7 No DNA control.The PCR product was identified by restriction digest. **b.** Lane 1 molecular weight markers. Lane 2 PCR product uncut. Lane 3-6 PCR product digested with Bam HI, Bsp120 I, Dra I and Pvu II respectively.

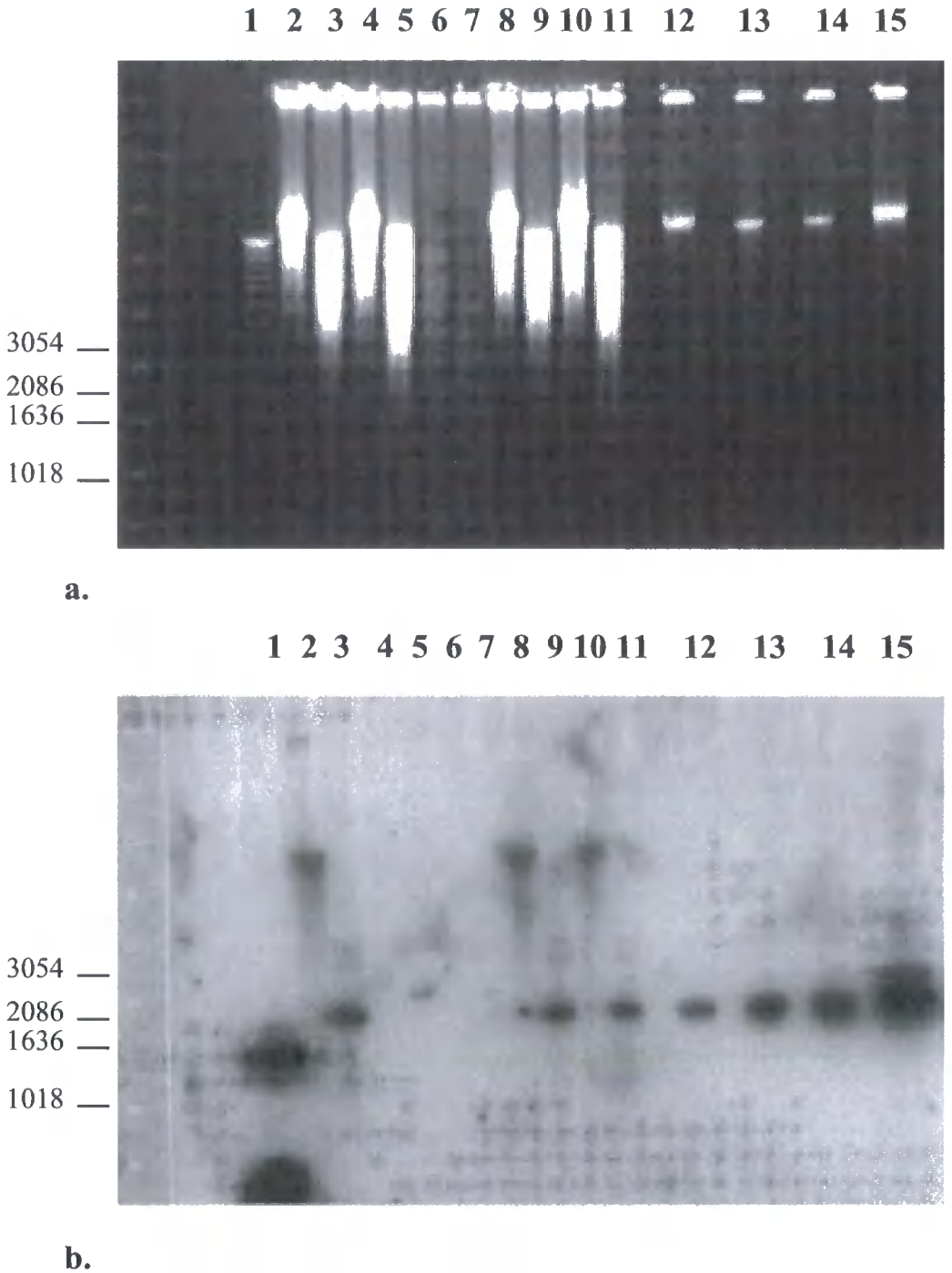


Figure B3.2.5 Southern Blot Analysis of the MBP-EGFP founder and F1 male pups. 10 microgrammes of genomic DNA from each mouse was digested overnight with the restriction enzyme BamHI. **a.** Uncut and digested DNA was then run on an agarose gel and blotted to nitrocellulose membrane. **b.** It was then incubated with a radiolabelled probe. Lane 1 molecular weight marker. Lane 2-11 male pup 1 to 4, founder 845-5, uncut and Bam HI digested DNA. Lane 12-15 control DNA corresponding to approximately 1, 5, 10 and 50 copies of the transgene in the diploid genome.

B4 Maintenance of the Transgenic Line

Due to the difficulties encountered during microinjection by the Karolinska Institute and the failure of incorporation requiring subsequent rounds of microinjection insufficient time remained for analysis of this transgenic mouse line. To maintain a colony of mice that at present would not be under active investigation would be prohibitively expensive. As an alternative the Karolinska Institute have frozen embryos derived from the male offspring of the original founder mouse for long term storage (see Appendix A3). In this way the transgenic line will be preserved until some future time when they may be analysed in full.

B4.1 Analysis of the Transgenic Mouse Line

To expand the transgenic population the founder mouse was mated with a wild type male. From the resulting litters tail biopsies were taken and sent to Durham for analysis. DNA was extracted from tail biopsies of four male pups of the F1 generation. PCR analysis identified two of these as positive for the EGFP gene and the whole MBP-EGFP insert. This was confirmed by Southern blot as described above (section B3.2, see Figure B3.2.5).

In order to estimate the number of copies of the transgene that had been incorporated into the genome of the founder mouse and her offspring, a number of standard amounts of the transgene DNA were investigated by Southern blot. The amount of DNA that corresponds to a single copy of the transgene within a stated amount of the full diploid genome can be determined. For example the transgene is 2330 base pairs in length within a single copy of the mouse diploid genome $6x10^9$ basepairs. In a total of 10μg of genomic DNA 3.9pg of the transgene fragment is equivalent to 1 copy of the transgene within the diploid genome.

$$\frac{6x10^9}{2330} = 2575107.3 \qquad \frac{10{,}000{,}000}{2575107.3} = 3.88\text{pg equivalent to one copy.}$$

Alongside 5μg of genomic DNA from both the founder 845-5 and the F_1 offspring, 3, 15, 30 and 150pg of the transgene fragment (Hind III/Afl II) were loaded amongst a small amount of wildtype genomic DNA. This was equivalent to 1.58, 7.9, 15.8 and 79.0 copies of the transgene respectively within 5μg of genomic DNA. Spectrophotometric analysis of the resulting bands found on the Southern blot (Figure B3.2.5) was used to estimate the copy number of the transgenic line.

Assuming that equal amounts of genomic DNA were loaded onto the agarose gel, comparison of the optical density of the band that relates to 1.58 copies can be made with the bands from the transgenic mice. It is noted that the DNA from the F_1 mouse 845-5/3 had degraded. The results show that approximately 2 copies of the transgene have incorporated into the genome of the founder 845-5, and that the same copy number has successfully been passed on to the offspring 845-5/1 and 845-5/4 (Figure B3.2.5).

Identity	Optical Density	Estimated Number of Copies
3pg of the transgene	0.263	1.58
15pg of the transgene	0.379	7.90
30pg of the transgene	0.418	15.8
150pg of the transgene	0.472	79.0
Founder 845-5	0.362	2.2
845-5/1 F_1	0.350	2.1
845-5/2 F_1	N/A	N/A
845-5/3 F_1	N/A	N/A
845-5/4 F_1	0.352	2.1

Table B4.1 Spectrophotometric determination of copy number. Analysis of the founder animal and its offspring by comparison of the density of the 3pg band. Limitations of the software cannot recognise increased density past a certain point. This means that a doubling of the optical density does not reflect doubling of the amount of standard DNA.

B5 Discussion

The successful production of a construct in which 1.3kb of the 5' sequence of the myelin basic protein promoter was coupled to drive EGFP expression has been shown. The construct was shown to be functional in one of the target cell types, primary Schwann cells, and previous studies using this promoter region have shown its activity in oligodendrocytes (Katsuki *et al.*, 1988; Kimura *et al.*, 1989; Miura *et al.*, 1989). In addition non-specific expression was seen in the control cell line, COS-7.

This construct was incorporated into the genome of a mouse line by microinjection at the Karolinska Institute in Sweden. A single founder was identified by PCR and confirmed by Southern blot. The transgene was shown to be transmitted to the offspring of that founder by Southern blot. Spectrophotometric analysis has estimated that two copies of the transgene had incorporated into the founder line. To date no further investigation of the animal has been made.

Initial analysis would need to determine if EGFP expression could be detected in the white matter of the CNS. This could be achieved either by *in vitro* culture of oligodendrocytes from the brains of neonatal animals (as described by McCarthy and de Vellis, 1980), or through direct sectioning of the brain. EGFP fluorescence may be lost during the cryopreservation technique and so expression may have to be determined with the use of anti-GFP antibodies.

Determination of the extent of expression both spatially and developmentally would also be necessary. Previously described transgenic mice employing the MBP promoter to drive reporter gene expression have encountered mosaic expression of the reporter gene product (Foran and Peterson, 1992).

A number of transgenic lines expressing reporter genes under the influence of the MBP promoter regions have been produced and described fully. They have been used to elucidate the exact pattern of expression of the MBP gene both temporally and spatially in the central nervous system. The specificity of the promoter has also been proved by its use to regulate the rescue of mutant mouse phenotypes. However there remains a

number of arguments as to why our particular animal model is worth continued investigation.

Recent work investigating the potential of gene delivery and expression systems in the central nervous system have taken advantage of the specific expression regulated by the MBP promoter and the ease of detection of the GFP gene (Chen *et al.*, 1998; Huang *et al.*, 1999). In a search for therapeutic systems of gene delivery to target cells an adeno-associated virus has been produced that contains the MBP promoter and the GFP gene. Chen *et al.* (1998) have shown that in this manner both *in vitro* and *in vivo* GFP can be successfully expressed in the target oligodendrocyte. This gives cause to believe that not only should the promoter sequence used be sufficient to describe specific expression, but also that the GFP itself is a suitable reporter gene that is capable of expression within the cell without toxicity.

To have created a mouse in which oligodendrocytes may express EGFP may prove to be a valuable tool in which to follow the dynamic interactions of glia and neurons. As EGFP can be readily visualised in live cells with no manipulation, it would provide a novel opportunity to study the development of neuron-glia relationships in the CNS.

The cerebellum contains a relatively small number of cell types organised in a stereotypic pattern. Much of the development of the interactions between these cells occurs postnatally making them accessible for study. Within the cerebellum oligodendrocytes can be identified and the full arbor of an individual cell may be seen. In organotypic and cerebellar thin slice cultures (Notterpek *et al.*, 1993) the structure and relationships between cell types are maintained. It has been shown that oligodendrocytes within this culture system retain the ability to myelinate (Raval-Fernades and Rome, 1998). This system of *in vitro* study, in conjunction with the MBP-EGFP mouse would provide a good model to study the associations of oligodendrocytes with neurons in the cerebellum.

Laser scanning confocal microscopy techniques could be deployed to study the process of myelination, three dimensional reconstructions of Z-axis series combined with time lapse microscopy could visualise the whole process. Questions could be addressed such

as in what way does the oligodendrocyte select neurons with which to interact? Oligodendrocytes myelinate many fibres, does this occur simultaneously or in a sequential manner?
The effect of factors that inhibit or stimulate myelination could be followed progressively over time, the observation of morphological changes and altered relationships with axons may suggest mechanisms of action.

Tissue culture techniques exist in which populations of the transgenic oligodendrocytes could be expanded. The mice in this way could provide a source of fluorescent green cells for transplantation experiments. In multiple sclerosis, and the animal model experimental allergic encephalomyelitis (EAE, Martenson, 1984), demyelinated plaques appear in the CNS and are thought to be the result of immunologic attack against the myelin producing cells. Around the periphery of these plaques healthy oligodendrocytes exist (Raine, 1983) that can spontaneously and often transiently repair the lesion. Transplantation of transgenic oligodendrocytes into the site of lesion in the EAE animal would allow a straight forward method of identifying donor cells and assessing their success at remyelination and the pattern of migration and division that has occurred following their insertion.

The focus of attention in the study of MBP rests on the central nervous system expression as it is this that is compromised following mutation of the gene. In the PNS compact myelin remains. Transgenic animals in which reporter genes are expressed with varying amounts of the MBP promoter fail to show expression in the peripheral nerve of these animals (Gow *et al.*, 1992; Foran and Peterson, 1992; Wrabetz *et al.*, 1998). Although weak expression has been reported when as much as 6.5kb of promoter region was utilised (Freidrich *et al.*, 1990). Some aspect of the promoter structure is thought to be absent within the studied region that regulates Schwann cell specific expression of the gene.
It has been shown here that 1.3 kb of the MBP promoter can direct expression of the reporter gene in primary Schwann cells *in vitro*. Other studies that have used this region of the promoter have studied its expression in neuronal/glial cell lines (Miura *et al.*,

expression in primary Schwann cells of the reporter gene is found. Whilst previous studies indicate that this regulatory region is insufficient to drive Schwann cell expression, our transfection studies indicate that it would be interesting to observe PNS myelin in the search for expression.

It should be noted however, that regulatory components of the *in vivo* environment may be absent within the tissue culture system, allowing tight restrictions on expression to be relaxed. However, this construct may prove a useful tool for the *in vitro* study of MBP expression and regulation in Schwann cells through either transient or stable transfection regimes.

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