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Myelin messenger ribonucleic acids : their association with the cytoskeleton and their subcellular distribution

This thesis is submitted as part of the requirements for the degree of Doctor of Philosophy.

CONTENTS

CONTENTS

ABSTRACT

This work presents an examination of the subcellular localization of the major myelin proteins of oligodendrocytes, and the messages that encode these proteins. The association of these messages with the cytoskeleton is also examined.

All messages investigated show some degree of association with the cytoskeleton; indeed messages encoding actin and tubulin are found exclusively bound to the cytoskeleton.

In general a specific myelin message and its protein counterpart are found in the same area of the cell. This suggests that these messages are translated close to their targetted site.

In most instances the messages being probed by *in situ* hybridization are being sought for the first time in cells grown in tissue culture, and in some instances messages are being probed for the first time ever.

In addition, the messages encoding DM-20 and PLP are found in different areas of the cell. As these are the products of a single gene, this suggests that there is a significant role to be played by differential splicing in individual cells.

The role of cytoskeleton-mRNA association in the targetting and assembly of myelin proteins is discussed.

ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CNP	2', 3' - cyclic nucleotide - 3' - phosphohydrolase
CNS	Central Nervous System
cpm	counts per minute
CSK	Cytoskeletal fraction
dATP	deoxyadenosine triphosphate
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
DME	Dulbecco's Modification of Eagle's Medium
DNA	Deoxyribonucleic Acid
EDTA	disodium ethylene diamine tetraacetate
EGTA	ethyleneglycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
FCS	Fetal Calf Serum
HBSS	Hanks Balanced Salt Solution
kb	kilobase
kDa	kilo Dalton
MAG	Myelin Associated Glycoprotein
MBP	Myelin Basic Protein
MOPS	Morpholinopropanesulphonic acid
mRNA	messenger Ribonucleic Acid
OD	optical density
PBS	Phosphate Buffered Saline
PLP	Proteolipid Protein
PNS	Peripheral Nervous System

PVP/.....

PVP	polyvinylpyrocarbonate
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecylsulphate
SOL	Soluble fraction of a cytoskeletal extraction
TLCK	N-p-tosyl-L-lysyl-chloromethane
UTP	Uridine triphosphate

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1. BASIC OUTLINE OF MYELIN

1.1.1. DISCOVERY AND PURPOSE OF MYELIN

1.1.1.1. DISCOVERY OF MYELIN

The brain of all higher vertebrates upon even a very casual inspection comprises white matter and grey matter. In addition the nerves of these animals are unmistakably brilliant white.

The white matter consists predominantly of closely packed cylinders with a white coating. These cylinders are axons, and the coating is myelin (Morell and Norton, 1980).

The term "myelin" was first used in 1864 by Rudolf Virchow, who derived it from the Greek word "myelos", meaning marrow. This was based upon his observation that myelin is particularly in the marrow or core of the brain (Morell and Norton, 1980).

1.1.1.2. FUNCTION AND STRUCTURE OF MYELIN

By 1878 Louis-Antoine Ranvier had suggested that myelin might be an electrical insulator, similar in function to the recently laid transatlantic cables. This function helped explain his earlier observation that the axonal membrane is exposed at gaps in the myelin sheath (Ranvier, 1871), known as the nodes of Ranvier.

The myelin sheath has a higher electrical resistance, and a lower capacitance, than the axonal membrane. Depolarisation of the membrane at the nodes of Ranvier generates a local circuit which cannot flow through the adjacent membrane. Instead the membrane is excited at the next node (Morell and Norton, 1980) (Fig. 1).

Since /...

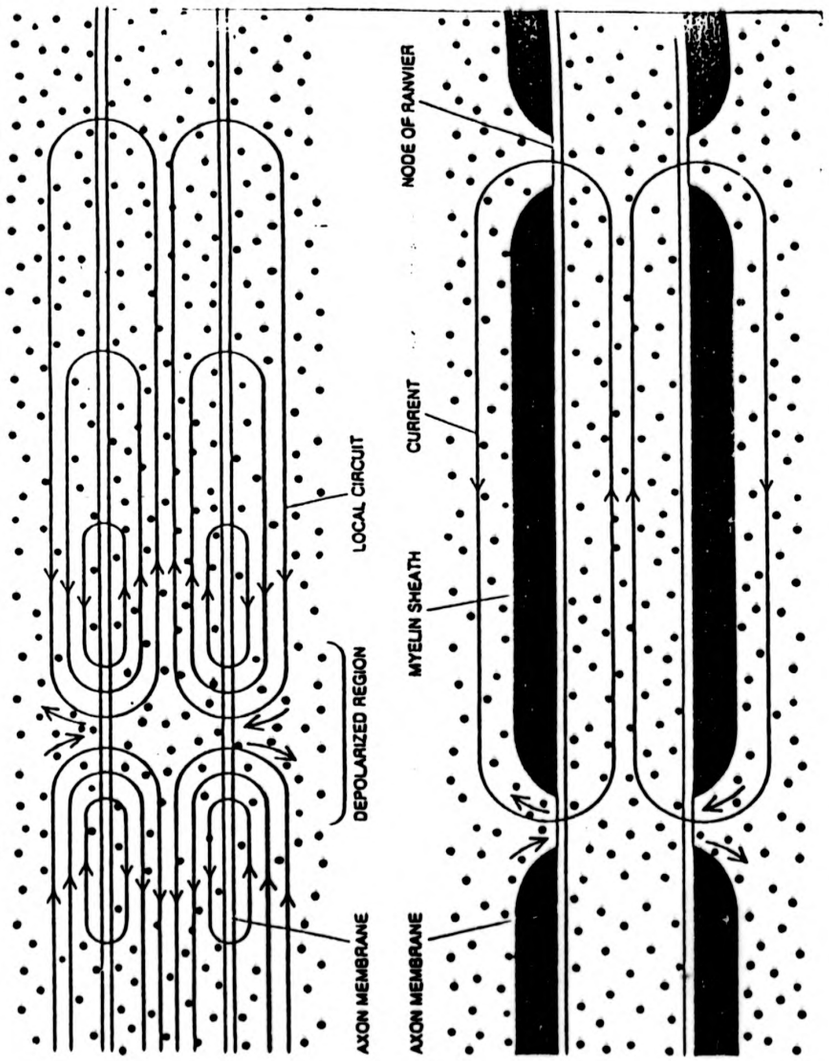


Figure 1

Figure 1. A schematic representation of the function of the nodes of Ranvier. The membrane becomes depolarized at the node of Ranvier and so a local circuit is generated which cannot flow through the adjacent membrane. Instead the membrane is excited at the next node. By this process the signal is able to move more rapidly along the axon.

The figure is taken from Morell and Norton (1980).

Since the excitation of the membrane is only from node to node, signals are conducted faster than would be the case in an axon of similar diameter which was devoid of myelin. Indeed, based upon the observation that the velocity of signal conduction by a bare nerve fibre increases with the square root of its diameter, it has been estimated that the human spinal cord would have to be several yards in diameter in the unmyelinated state to conduct signals at the same speed as signals are conducted in the myelinated spinal cord (Morell and Norton, 1980).

When viewed in cross-section, myelin sheaths clearly display a structure like a spiral, which may be thought of as alternating layers of protein and lipid (Morell et al, 1989). This concept is further implied by X-ray studies which suggest units of 80Å, which could be explained as two 15Å protein layers and a 50Å layer with primarily lipids (Kirschner et al, 1984). This lipid layer is known to also traverse the myelin bilayer.

Further evidence for lipid-protein layers is obtained from elecron microscope studies. Here myelin appears as a series of alternating dark and less dark lines (proteins) interupted by unstained areas (lipids). The darker line, or major period line, is the fused inner protein coating of the cell membrane. The less dark, or intraperiod line represents the closely apposed outer protein coating of the original cell membrane (Colman et al, 1982) (Fig. 2).

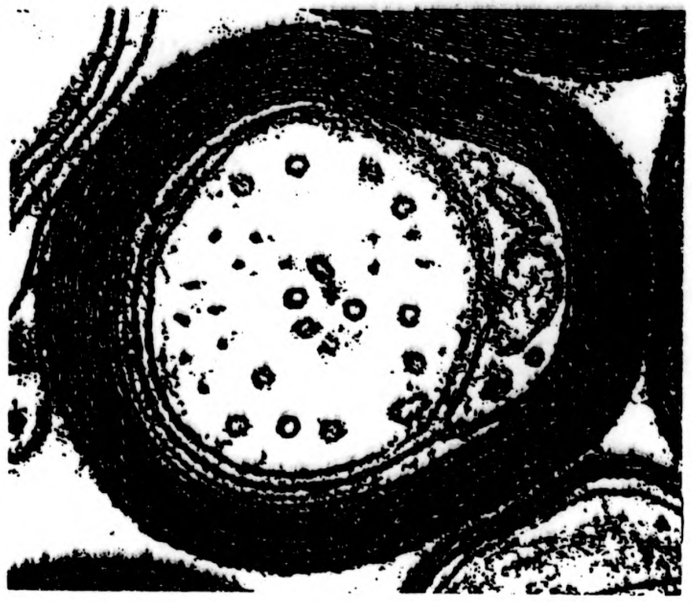


Figure 2

Figure 2. A myelinated nerve viewed in cross-section. The myelin appears as a series of alternating dark and less dark lines interupted by unstained areas. The dark areas are proteins and the unstained areas are lipids.

Size Bar = 100nm

The figure is taken from Colman et al (1982).

1.1.2. MYELINATING CELLS

In higher vertebrates two distinct types of myelin exist, that of the central nervous system (CNS) and that of the peripheral nervous system (PNS). In the PNS the Schwann cell is responsible for myelination, and in the CNS this role is performed by the oligodendrocyte.

Phylogenetically Schwann cells appear before oligodendrocytes and this is also true for PNS myelin and CNS myelin. This is based on the observation that annelids and crustaceans have only Schwann-cell myelination (McAlear et al, 1958).

Ontogenetically the Schwann cell is of ectodermal origin, migrating from the neural crest into the PNS (Harrison, 1924). By contrast oligodendrocytes differentiate from cells of the primitive neural tube (Hinds and Ruffett, 1971).

It is believed that the precursors of oligodendrocytes are also the precursors of astrocytes and neurons. Furthermore oligodendrocytes and type-2 astrocytes have been shown to descend from a common bipotential progenitor cell; the oligodendrocyte-type-2-astrocyte (O-2A) in cultures of optic nerve glial cells (Raff et al, 1983).

In addition to being from different origins, the oligodendrocyte and Schwann cell lines myelinate in different manners, and do not commence myelination at the same time (Raine, 1984).

Oligodendrocytes initiate myelination some days after the onset of PNS myelination. At this time the oligodendrocyte's processes form loose cups around segments of the axon. One lip of the cup is then covered by the other lip and subsequently the inner tounge is wrapped around the axon and covered by additional layers. This leads to a rapid compaction /...

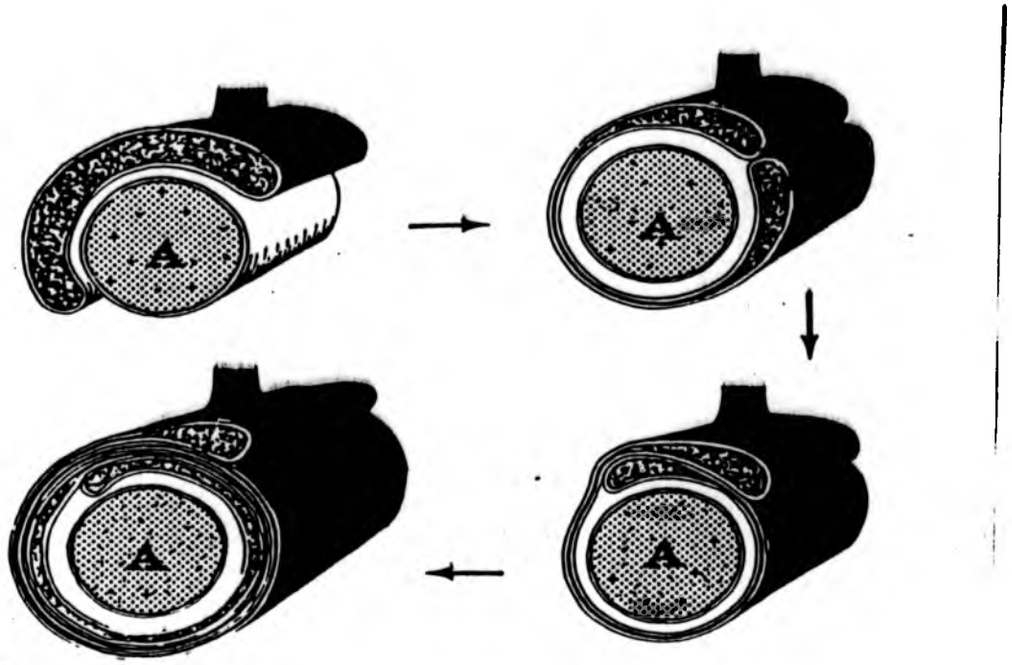


Figure 3

Figure 3. A schematic cross-section of an axon being myelinated by an oligodendrocyte. The processes of the oligodendrocyte form loose cups around segments of the axon. One lip of the cup is then covered by the other lip and so the inner tongue is wrapped around the axon and covered by additional layers. This in turn leads to CNS myelin compaction.
The figure is taken from Peters and Vaughn (1970).

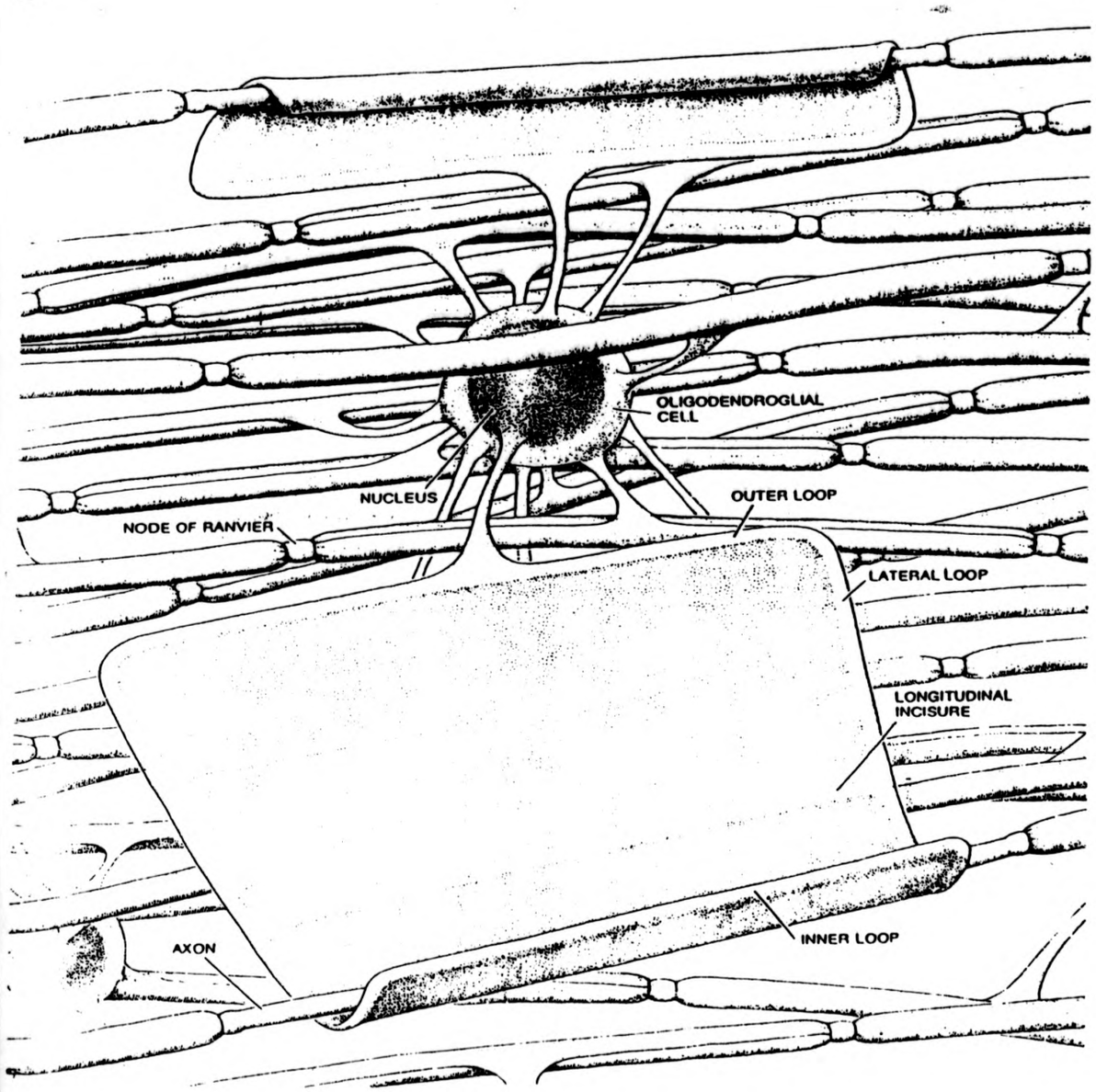


Fig. 4

Figure 4. A schematic representation which demonstrates the fact that oligodendrocytes have the capacity to myelinate a number of different sections of different axons.
The figure is taken from Morell and Norton (1980).

compaction of the CNS myelin (Peters and Vaughn, 1970) (Fig. 3).

Initially it is fairly simple to demonstrate association of a particular oligodendrocyte with a particular myelinated axon. However as time elapses, the oligodendrocyte becomes associated with several sheaths (Peters, 1964) (Fig. 4). It has been estimated that one oligodendrocyte must often myelinate about 40 segments of axons (Peters and Vaughn, 1970). The myelination process in the PNS commences when the Schwann cell and axon lie side-by-side. The Schwann cell (if viewed in cross-section) behaves like a macrophage engulfing a foreign body and one cell ridge tucks under the other, so creating spiralling and therefore myelination. (Raine, 1984). Each cell myelinates only one section of a single axon (Fig. 5 & 6).

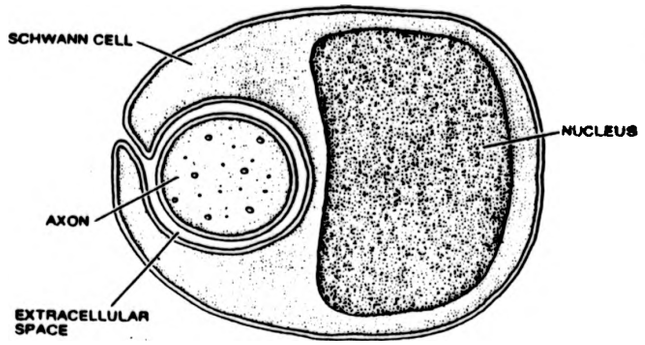


Figure 5

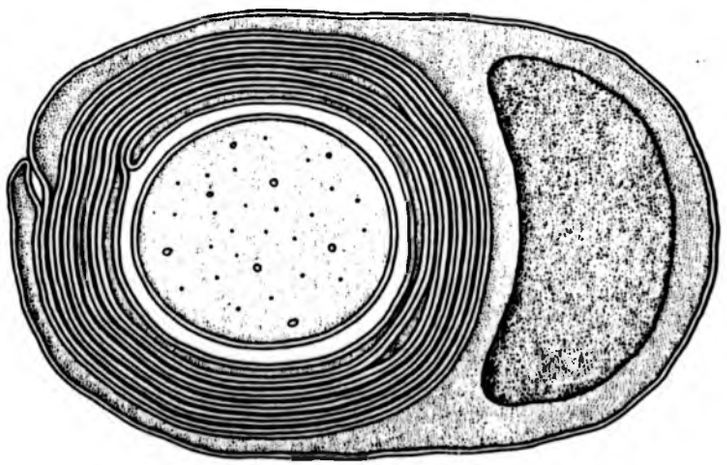


Figure 6

Figure 5. A schematic cross-section of the initiation of myelination of an axon by a Schwann cell. The figure is taken form Morell and Norton (1980).

Figure 6. A schematic cross-section ofan axon which has been completely, or almost completely, myelinated by a Schwann cell. Note the inner tongue of the sheath which has spiraled around the axon to create multiple layers.

TABLE 1

	Human Myelin	Bovine Myelin	Rat Myelin	Total Rat Brain
Lipid: Protein	70:30	25:25	70:30	57:43
Cholestrol	27.7	28.1	27.3	23.0
Cerebroside	22.7	24.0	23.7	14.6
Sulphatide	3.8	3.6	7.1	4.8
Total Galactolipid	27.5	29.3	31.5	21.3
Ethanolamine Phosphatides	15.6	17.4	16.7	19.8
Lecithin	11.2	10.9	11.3	22.0
Sphingomyelin	7.9	7.1	3.2	3.8
Phosphatidylserine	4.8	6.5	7.0	7.2
Phosphatidylinositol	0.6	0.8	1.2	2.4
Total Phospholipid	43.1	43.0	44.0	57.6

Table 1. The composition of myelin in terms of lipids, compared with the total membrane content of the rat brain. No lipid is myelin specific, although the relative abundance of certain lipid is quite characteristic to myelin when compared with other types of membrane.

1.2. COMPOSITION AND EVOLUTION OF MYELIN

1.2.1. MYELIN COMPOSITION

1.2.1.1. MYELIN LIPIDS

Myelin has a very high lipid: protein ratio, relative to other cell-surfaces or intracellular membranes. In addition myelin has a highly characteristic lipid pattern, unlike any other membrane, although no lipids are exclusive to myelin (Norton and Cammer, 1984). The lipid pattern of myelin and total brain membranes is summarised in Table 1.

1.2.1.2. MYELIN PROTEINS

As well as lipids myelin contains proteins. While the number of proteins in myelin is unquantified, the number of major proteins is relatively small. Some proteins are exclusive to myelin and others are found in other loci. Of the myelin specific proteins, some are common to both PNS and CNS myelin, and some are specific to one or the other. Table 2 summarises the properties of the major proteins found in CNS myelin; proteolipid protein (PLP), myelin basic protein (MBP), myelin-associated glycoprotein (MAG), 2',3'-cyclic nucleotide-3'-phosphohydrolase (CNP), actin and tubulin.

Of these proteins MAG has the best defined protein function. MAG is involved in the interaction between cells, both oligodendrocyte-oligodendrocyte interactions, and also oligodendrocyte-axon interactions (Poltorak *et al*, 1987). In addition,in Schwann cells at least, there is evidence that MAG plays an early role in the process of myelination (Owens and Bunge, 1989).

Furthermore /...

TABLE 2

	PLP/DM20	MBP	MAG	CNP	Actin	Tubulin
Protein Size (kDa)	20-25 (g)	14, 17, 18.5, 21.5 (i)	100 (a)	46 & 48 (d)	43 (m)	50
Number of Forms	2 (g)	5 or more (i)	2 (a)	2 (d)	Multiple (k)	Multiple (k)
Chromosomal Locus	Human Xq13-Q22 (f)	Human 18q22-q23 (h)	Human 19 Mouse 7 (b)	Mouse 11 (c)	Multiple (k)	Multiple (k)
Message Size (kb)	1.6 & 3.2 (e)	2.2 (i)	3.5 (a)	2.4 & 2.8 (c)	2.0 (k)	1.8 & 2.4 (l)
Percentage of CNS Myelin Proteins	50% (e)	25% (j)	1% (a)	4% (d)	-	-
CNS/PNS	CNS	Both	Both	Both	Both	Both

(a) Arquint et al (1987)
(b) Barton et al (1987)
(c) Bernier et al (1988)
(d) Vogel and Thompson (1988)
(e) Milner et al (1985)
(f) Diehl et al (1986)
(g) Van Dorsselaer et al (1987)
(h) Kamholz et al (1987)
(i) Newman et al (1987)
(j) de Ferra et al (1985)
(k) Cleveland et al (1980)
(l) Villasante et al (1986)
(m) Nudel et al (1983)
- Undetermined

Table 2. A summary of the major physical properties of the major proteins in the mammalian central nervous system. Detailed in this table are the sizes of these proteins in kiloDaltons, the number of different forms of these proteins present - both as a result of differential splicing and also due to multiple copies a specific gene family, the size of messages encoding these proteins, the chromosomal locus of the respective genes, the relative abundance of the proteins in terms of total myelin protein, and whether the protein is specific to the CNS, or if it is also found in the PNS.

TABLE 3

	PNS MYELIN				CNS MYELIN						
	MBP	MAG	P_o	P_2	MBP	MAG	CNP	36K	PLP	DM-20	P_o
Elasmobranchii	+	+	+	-	+	-	-	-	-	-	+
Chondrostei	+	+	+	-	+	-	-	-	-	-	+
Holostei	+	+	+	-	+	-	-	+	-	-	+
Teleostei	+	+	+	-	+	-	-	+	-	-	+
Polypteri	+	+	+	-	+	-	-	+	+	-	+
Dipnoi	+	+	+	-	+	-	-	-	+	-	-
Amphibia	+	+	+	-	+	+	+	-	+	-	-
Reptilia	+	+	+	+	+	+	+	-	+	+	-
Aves	+	+	+	+	+	+	+	-	+	+	-
Mammalia	+	+	+	+	+	+	+	-	+	+	-

\+ = Present
\- = Absent

Table 3. This table shows the distribution of the major myelin proteins between the PNS and CNS of a number of different classes of vertebrates. It is quite clear from this evolutionary construct that PLP is the precursor of DM-20, rather than the reverse. In addition P_o is common to both the CNS and PNS of certain classes of fish, but in all amphibians, reptiles, birds and mammals P_o is specific to the PNS. In addition certain classes of fish have a protein known as 36K which is unique to these organisms.

The table is based on Waehneldt et al (1986).

Furthermore it has been found that there is an interaction between MBP and PLP (Edwards et al, 1989). This discovery was made using a microtitre-well binding assay where iodinated PLP bound to MBP, but not to other CNS myelin proteins. In addition the amount of PLP binding to Histone H4 was much less than that of the PLP-MBP association, suggesting that the interaction is highly specific.

1.2.2. EVOLUTION OF MYELIN

In terms of evolution it appears that Schwann cells and PNS myelin evolved before oligodendrocytes and CNS myelin. This is based upon the observation of McAlear et al (1958) that annelids and crustaceans have only Schwann cell myelination. This work relied upon morphological appearance to determine cell type, rather than use of immunocytochemistry.

Table 3 (based on Waehneldt et al, 1986) illustates the components of myelin in various vertebrate classes.

In addition to the information in Table 3 it should be noted that P_2 is found in the spinal cord oligodendrocytes of rabbits (Gillespie et al, 1989; Trapp et al, 1987).

This quite clearly illustrates that certain proteins are common to all forms of myelin. It also shows that others emerge during evolution. In addition it is interesting to note that PLP is the precursor of DM-20 rather than the other way round, as had been suggested (Laursen et al, 1983).

The 36K protein found in certain fish is not recognised by any antibodies raised against the other myelin proteins. This non-recognition is observed for antibodies raised against other fish proteins and also for proteins obtained from other classes of vertebrates.

1.2.3. USE OF MYELIN MUTANTS AS A MEANS OF ELUCIDATING PROTEIN FUNCTION

Although the protein sequence, and even the DNA sequence, of a number of individual myelin proteins is currently known, very little is known about the function of each individual protein as regards its role within the myelin membrane, with the exception of MAG (see Section 1.2.1.2.).

For example it is well documented that CNP has the enzymic property of hydrolysing 2'3' cyclic nucleotides, but owing to the absence of 2',3'-cyclic nucleotides in the brain, it is difficult to present a case for this as its sole function.

One strategy to determine the function of a protein is to study myelin obtained from animals which are mutant for the particular protein of interest. To date a number of mutations which specifically affect myelin have been documented. Such mutations have been found in dogs (Griffiths *et al*, 1981), rats (Dentiger *et al*, 1982), humans (Seitelberger, 1970) and particularly in mice.

By determining the precise genetic defect for each mutation, it may be possible to study how the remaining myelin components interact with each other in the absence of a specific protein, or with a modified form of that protein. Such studies in turn may illustrate the role of each protein within myelin, and hence lead to a better understanding of myelin biosynthesis.

Some mouse mutations have been well classified, to the level of the individual nucleotide, others have been localised to a specific gene and some are very poorly defined. Examples of each category are; jimpy, which is a point mutation in the PLP gene (Macklin *et al*, 1987); shiverer, which has been documented /...

been documented as a partial MBP gene deletion (Roach et al, 1985); and quiverer which is suspected as a MAG mutation, but has not yet been proven to be so (Sutcliffe, 1987).

To date the most promising research using mutants, as regards determining protein function, is that of the shiverer mouse. Shiverer is an autosomal recessive mutation which causes greatly deficient CNS myelin (Bird et al, 1978) but the PNS myelin sheath is largely unaffected (Rosenbluth, 1980). These mice display a general tremor by around 10-14 days, which gradually gets worse. By a few months old convulsions commence, leading to death at about 90-150 days (Chernoff, 1981). The shiverer phenotype has been corrected using transgenic shiverer mice homozygous for an introduced wild type MBP gene (Readhead et al, 1987). More recently it has been shown that only the 14kDa protein is required in transgenic mice to correct the mutant phenotype (Kimura et al, 1989).

When viewed ultrastructurally CNS myelin appears as loose cytoplasm-filled whorls in shiverer mice (Kirschner and Ganser, 1980). The reason the PNS is less severely affected is thought to reflect the fact that MBP is relatively less abundant than in the CNS, and also due to a partial, incomplete homology between sections of MBP and P_o. This homology, is not sufficient to suggest a common evolutionary origin (Lemke and Axel, 1985). Hence MBP is thought essential for efficient myelin compaction, at least in the CNS (Kirschner and Ganser, 1980).

In addition the mld^- allele of the shiverer gene, in the homozygous form has been found to lead to abnormally high levels /...

levels of transcripts encoded by both the CNP and MAG genes (Matthieu *et al*, 1990). When *in situ* hybridization studies have been performed on homozygous *mld*$^{-}$ mutants using MBP probes the messages were found generally over the nucleus and that they were particularly unstable (Fremeau and Popko, 1990). Jimpy mice have a mutant PLP gene were a single base change leads to a 74bp exon not being included in the mature mRNA, having been spliced out instead. This in turn not only results in the removal of around 25 amino acid residues from the translated protein, but also leads to a frameshift mutation for all subsequent bases. Performing *in situ* hybridization on jimpy mice using an MBP probe results in MBP messages being found almost exclusively around the nucleus of the oligodendrocytes (Verity *et al*, 1990). It is proposed that this observation reflects a truncation or absence of any processes of note in the mutant cells. Hence it has been tentatively proposed that PLP may have some role to play in helping with subcellular transportation of MBP encoding messages.

In summary myelin specific mutants have only proved slightly successful in helping understand the function of the major myelin proteins, but this reflects the importance of not treating each protein as a separate entity, and highlights the importance of studying their interactions.

However mutation studies have suggested a role for MBP in myelin compaction and also a possible role for PLP in subcellular transportation of the messages encoding the MBP proteins.

1.3. MYELIN mRNA

1.3.1. RNA MATURATION

The process of DNA transcription to produce RNA, which in turn is translated to give a protein, is a highly complex phenomenon which can be broken down into several stages. The newly transcribed RNA must be 5' capped, 3' polyadenylated, spliced and transported to the correct site of translation.

5' capping of RNA involves adding a 7-methylguanosine residue, linked to a triphosphate molecule to the 5' end of the RNA (Kozak, 1978).

Polyadenylation is the sequential addition of adenosine residues until a poly A tail of around 200-300 nucleotides is generated (Manley, 1988).

RNA splicing is common to almost all pre-mRNAs of the higher eukaryotes, and is the removal of internal sequences (introns) which interupt the coding domains (exons) (Crick, 1979). Quite obviously it is vital that intron removal is precise to the single nucleotide level. If even only one nucleotide is left, or one too many removed, a frameshift mutation will result.

Exactly why the process of splicing should occur is as yet unknown, but it is possible that introns play a role in the evolution of proteins. This hypothesis is based upon the evidence that in the fibronectin gene there has been shuffling of exons during evolution (Patel *et al*, 1987).

The problem of correct splicing is compounded even further by the process of differential splicing. Differential splicing involves certain exons being left in one mRNA, but being absent from another. Hence it is critical that the correct exons /...

exons are present in any one message. Differential splicing is of particular interest when studying the molecular biology of myelin proteins as a number of the proteins are the product of this process; mouse MBP is known to have at least 5 differentially spliced products (Newman et al, 1987); CNP is thought to have 2 differential products (Bernier et al, 1987); MAG has 2 products (Lai et al, 1987); and PLP has 2 products (Trifilieff et al, 1985).
The exact function of these different differential splicing products has yet to be determined.

1.3.2. MBP DIFFERENTIAL SPLICING PRODUCTS

Both mouse and rat species have been known to have at least four forms of MBP for some time (Yu and Campagnoni, 1982; Agrawal et al, 1982). In both species the proteins are 14kDa, 17kDa, 18.5kDa and 21.5kDa. By using a rat cDNA clone encoding the smallest MBP to screen a mouse genomic library, five exons were detected. In addition using a synthetic oligonucleotide a sixth exon was picked up, corresponding to the DNA encoding the extra amino acids present in the 18.5kDa species (Takahashi et al, 1985).
Since the work of Takahashi et al (1985) a seventh coding exon has been found (de Ferra et al, 1985). Two additional 5' untranslated exon sequences have also been detected (Kitamura et al, 1990). These have been designated exon 0 and exon 1A; exon 1A lying directly beside the normally designated exon 1 (now exon 1B) and exon 0 lying at least another 25kb upstream. No function is proposed for messages containing these two additional exons; M41-MBP messages.
However /...

TABLE 4

	Protein Size (kDa)	Exons Present	Exons Absent
Mouse/Rat	14	I, III, IV, V, VII	II, VI
	17	I, II, III, IV, V, VII	VI
	17	I, III, IV, VI, VII	II, V
	18.5	I, III, IV, V, VI, VII	II
	21.5	I, II, III, IV, V, VI, VII	-
Human	17	I, III, IV, VI, VII	II, V
	18.5	I, III, IV, V, VI, VII	II
	20	I, II, III, IV, VI, VII	V
	21.5	I, II, III, IV, V, VI, VII	-

Table 4. A summary of the exons detained, and those spliced out of the MBP messages in order that the various different MBP proteins may be produced. It is of particular interest to note that the proteins which result are not the same size in both humans and rodents.

However it is clear that the M41-MBP messages are relatively more abundant in the spinal cord than in the brain, implying regional variation within the CNS (Kitamura et al, 1990). By omitting varying combinations of exons it is possible to generate all reported MBP products (Newman et al, 1987; Roth et al, 1987). The exons present in any particular MBP protein are summarised in Table 4.

In mice a minimum of five MBPs are present (Newman et al, 1987) and possibly as many as eight could be present if all possible combinations are utilised. Due to the varying absence/presence combinations it is impossible to study any difference in either mRNA or protein distribution in situ with currently available technology, but the possibility for in situ hybridization study of various MBP encoding messages is discussed in Section 4.

1.3.3. PLP AND DM-20 DIFFERENTIAL SPLICING PRODUCTS

Unlike the MBP gene, the PLP gene has only two protein products; PLP and DM-20. The entire gene contains seven potential exons (Diehl et al, 1986). The exact portion of message absent in DM-20 has long been the subject of debate.

Initially Lees et al (1979) suggested that residues 197-267 were absent, but this hypothesis was disproved (Trifilieff et al, 1985). This group counter-proposed that residues 100-140 ($\pm$ a few residues) are absent, based upon a combination of chromatographic separations in organic solvents. To further their hypothesis Trifilieff et al made a synthetic peptide which corresponded to residues 117-129. Using this tridecapeptide they raised an antibody which they hoped would be PLP specific and unable to detect DM-20 (Trifilieff et al, 1986). Using this /...

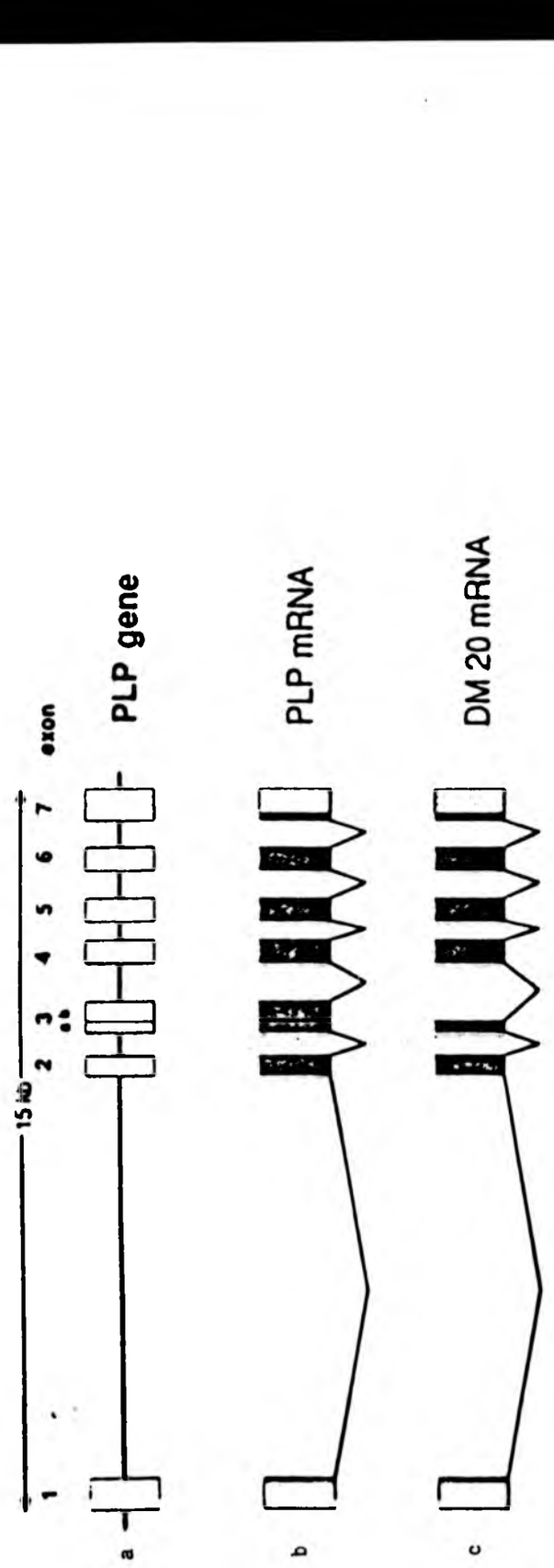


Figure 7

Figure 7 . A schematic representation of the PLP gene showing regions of the gene which are both transcribed and translated.

a) The entire stretch of 15kb is transcribed to give hnRNA. The blocks show stretches - exons - which are available to become part of mature messages. The lines show introns which will be spliced out of the mature message.

b) The dark blocks show the sequences of mRNA which will be translated to give PLP, the white blocks are the 5' untranslated and 3' untranslated sequences of the PLP message.

c) The dark blocks show the sequences of mRNA which will be translated to give DM-20, the white blocks are the 5' untranslated and 3' untranslated sequences of the DM-20 message.

this antibody on Western blots, their theory was proved correct adding further weight to their proposed deletion site.
Eventually the precise differentially omitted region was determined by use of DNA sequencing in both rodents (Nave et al, 1987) and in humans (Simons et al, 1987). This was found to be residues 117-151 in humans and 116-150 in rodents.
In the light of previous work on PLP exon determination, (Diehl et al, 1986) this discovery is particularly interesting.
Unlike conventional RNA differential splicing where entire exons are either absent or present, only part of an exon is missing; the 3' 105 nucleotides of exonIII (referred to as exonIIIB) (Fig. 7).
By synthesising an oligonucleotide specific to exonIIIB, it should be possible to study specifically the PLP encoding mRNA in a manner similar to the tridecapeptide-specific antibody used by Trifilieff et al (1986).
In a similar light, by synthesizing an oligonucleotide corresponding to the 3' end of exonIIIA and the 5' end of exonIV it should be possible to detect only DM-20 encoding mRNA. This is assuming the presence of exon IIIB should prevent this oligonucleotide successfully hybridizing to the PLP specific messages (Fig. 8).

Figure 8. The DNA sequence of exon III of the rat PLP gene. Exon IIIB which is spliced out of the DM-20 encoding message is underlined. This sequence is specific to the PLP encoding messages. The remaining sequence which is not underlined - exon IIIA - is common to both PLP and DM-20 messages.

1.4. RNA PROCESSING

1.4.1. RNA TRANSPORTATION FROM THE NUCLEUS

Evidence exists that mRNA is transported from the nucleus outward (Davis et al, 1987). This work was carried out using neurons incubated in ^{3}H-uridine followed by incubations for varying times in non-radioactive uridine. With time, progressively more distal areas of the neurons were found to contain radioactive uridine. This suggests that the pool of unincorporated uridine, together with the newly transcribed messages, are located in and around the nucleus. Only gradually can they be transported to the peripheral regions of the cell.
Using cytoskeletal extractions (removal of the cytosolic component of the cell, using non-ionic detergents) it was clear that many messages are retained attached to the cytoskeleton. In addition these cytoskeletally attached messages were found through-out the entire cell.
Hence it was suggested that there is a role played by the cell's cytoskeleton in RNA transportation.
In order that the mRNA is able to become associated with the cytoskeleton, it must have left the nucleus. It is generally accepted that there is more mRNA produced than ever reaches the cytoplasm (Schroder et al, 1987). For a message to leave the nucleus it must be fully mature; polyadenylated, 5' capped, fully spliced and if necessary methylated (Jacobs and Birnie, 1982; Otegui and Patterson, 1981; Kindas-Mugge and Sauermann, 1985).
Transportation from the nucleus is via the nuclear pore complexes (Maul, 1977) and is an ATP-dependent process (Wunderlich and Herlan, 1977). This can be viewed as occurring /...

occurring in three distinct stages (Schroder et al, 1987)

i) the mRNA is released from the internal nuclear matrix structure

ii) mRNA is translocated through the nuclear envelope pore complex

iii) the transported mRNA associates with the cytoskeletal elements.

Once mature, the mRNA has been transported out of the nucleus, association with the cytoskeleton is immediate (Jeffrey, 1982) and is thought to be a pre-requisite for mRNA translation (Bonneau et al, 1985; Moon et al, 1983).

1.4.2. RNA TRANSLATION

Translation is the process by which new polypeptides - and in turn proteins - are synthesized from their corresponding mRNA. In order that translation may proceed it is essential that the mRNA is associated with ribonucleoprotein particles known as ribosomes (Wool, 1979; Lake, 1981). Ribosomes are composed of one large and one small sub-unit which dissociate reversibly after each round of protein synthesis. These subunits are then recycled to create fresh ribosomes.

However the majority of the mass of a ribosome is composed of RNA rather than protein. This ribosomal RNA (rRNA) is thought to contain regions of complementarity to selected sequences of both the mRNA and tRNA. Hence the rRNA plays a vital link in the process of mRNA-tRNA recognition. In essence ribosomes can be thought of as protein-manufacturing machines which bring together the necessary cellular components for mRNA translation. When the initiation codon (AUG) on the mRNA is paired with a complementary /...

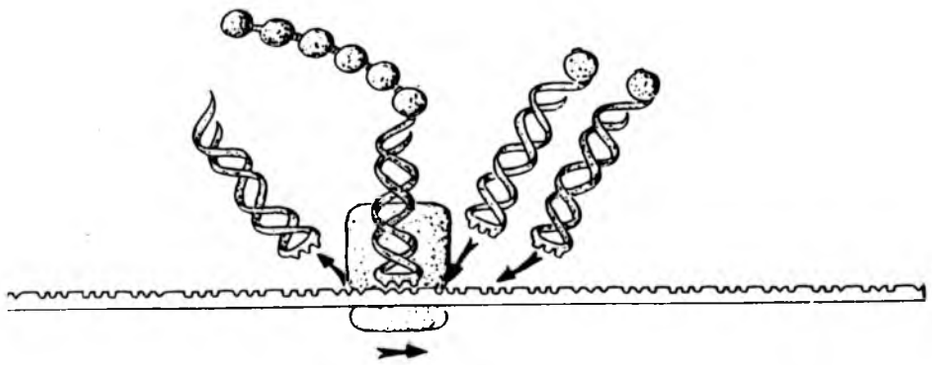


Figure 9

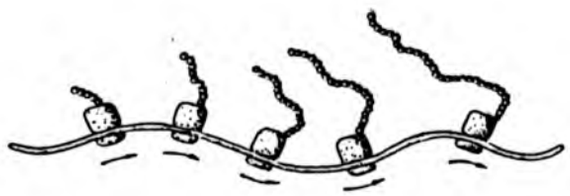


Figure 10

Figure 9. A schematic representation of a ribosome moving along a message synthesizing a new polypeptide. The tRNA molecule which has an amino acid attached becomes associated with the ribosome. The amino acid part of the molecule then becomes incorporated onto the nascent polypeptide and the tRNA molecule is free to dissociate from the ribosome.
Figure from MacLeoid and MacThomais (1976).

Figure 10. Many ribosomes can be associated with any one particular message at any time. Usually the ribosomes are approximately 80-100 nucleotides apart.
Figure from MacLeoid and MacThomais (1976).

a complementary tRNA molecule this starts a new polypeptide chain. The codon AUG encodes a methionine residue and so the nascent polypeptide will always start with methionine. Using energy generated from GTP hydrolysis, the ribosome moves along the mRNA in the 5' to 3' direction elongating the new polypeptide as it does so by addition of one amino acid at a time (Figs. 9 &10).

As elongation proceeds a tRNA molecule which bears a complementary codon to the next codon on the mRNA becomes associated with the ribosome. The amino acid associates with this new tRNA molecule is then added to the carboxyl-terminal end of the elongating polypeptide chain. Protein synthesis is terminated when one of the three stop codons is encountered (UAA, UAG, UGA). This whole process of protein synthesis is remarkably fast, requiring only around 20-30 seconds to synthesize a 40kDa protein from beginning to end.

The progression of translation is helped by the phenomenon known as codon redundancy, whereby the same tRNA molecule is often able to recognise a number of codons. For example the mRNA codons CCU, CCC, CCA and CCG may all be recognised by the same tRNA molecule- which results in addition of a proline residue to the polypeptide.

Almost as soon as one ribosome is sufficiently 3' of the AUG start codon to not interfere with another ribosome, a new ribosome will attach to the message and begin translation. Hence an actively translated mRNA will generally have many ribosomes associated with it at any one time. Such a polyribosomal status leads to several ribosomes being present, around 80 nucleotides apart, on the same mRNA molecule (Rich, 1963).

Polysomes can be caterogised into two classes; "free" and "membrane-bound" polysomes. Membrane-bound polysomes are involved in translation on the rough endoplasmic reticulum membrane. Free polysomes on the other hand tend to be found in association with the cytoskeleton. Hence the term "free" does not imply that these polysomes are free-floating, but rather describes them as being free from the rough endoplasmic reticulum membrane (Lewin, 1983). In addition it is worthwhile noting that free polysomes tend to be translation site of intracellular proteins, and that proteins destined to be exported from the cell tend to be translated on bound polysomes (Walter et al, 1984). However this observation by Walter et al does not imply that exported proteins are translated exclusively on the rough endoplasmic reticulum. Likewise they do not imply that translation on free polysomes will result in the protein being purely retained within the cell. Translation, both on free and bound polysomes, is performed in association with a number of proteins. In particular the proteins associated with messages tend to be either components of the cytoskeleton, or proteins associated with the cytoskeleton. This association of messages with the CSK is discussed in more detail in Section 1.4.4.

In conclusion, mRNA translation is the process of synthesis of proteins. This process is performed in a highly structured manner as the messages do not float free in the cytoplasm, but rather they are associated with ribosomes and also other proteins within the cell, most typically those involved in the structural cytoskeletal domains.

1.4.3. RNA LOCALIZATION

The idea that RNA is found throughout the entire cell is now a generally accepted concept (see Section 1.4.1.). Having established that this is true for RNA in general, it would be interesting to view a specific message and determine its sub-cellular locus; is it confined to one particular part of the cell, or is it generally located throughout the cytoplasm. The idea that a specific mRNA may be located in a specific part of the cell stems from the observation that some proteins are differentially localised e.g. actin filaments are found at the periphery of cells (Ishikawa et al, 1969; Buckley, 1981; Small, 1981; Stossel, 1984) but microtubules assemble from the nucleus towards the cell boundaries (Brinkley et al, 1975; Osborn and Weber, 1976; Brenner and Brinkley, 1984).

The first study of spatial distribution of specific mRNA found a difference in the sub-cellular localization of the messages encoding actin and tubulin (Lawrence and Singer, 1986), as determined by use of in situ hybridization. This study has been further expanded to the electron microscope level (Singer et al, 1989). The actin message is found primarily in the peripheral areas of the fibroblasts, but messages for tubulin are found more commonly around the centre of the cell. Thus Lawrence and Singer (1986) illustrated that there is some degree of spatial distribution for at least some of the messages.

The explanation proposed for this non-uniformity is that some messages are transported to the region their protein counterpart usually occupies within the cell. This proposal seems quite logical for messages translated on free polysomes as they are able to associate with the cytoskeleton throughout the cell. However /...

However membrane-bound ribosomes are found associated with the endoplasmic reticulum, which is generally found around the nucleus (Terasaki et al, 1986), are less likely to be found distributed around the cell.

Hence it seems plausible to assume that membrane-bound ribosomes will be found in the centre of the cell, but free polysomes will be found through-out the cell. In addition messages translated on one form of ribosome will be restricted accordingly in their distribution.

1.4.4. ASSOCIATION OF mRNA WITH THE CYTOSKELETON

Having established that a particular species of RNA may show spatial segregation, it is worthwhile contemplating how this segregation happens. Unfortunately no definite model of mRNA segregation has been proposed, but a number of factors involved in the mechanism have been discovered.

First of all, it has been shown that the vast majority of polyribosomes remain associated with the cytoskeletal framework after extraction with 0.5% Triton X-100 (Fey et al, 1986). It has been suggested that this cytoskeletal association is necessary for successful RNA translation on free polysomes. Since such a high degree of association between mRNAs and the cytoskeleton exists, it is reasonable to assume that spatial segregation must be performed on messages which are bound to the cytoskeleton. This does not automatically imply that cytoskeletal association is a prerequisite for segregation, but is merely an observation that messages associated with the cytoskeleton can be segregated.

However the evidence presented in Davis et al, (1987) suggests that association of the messages with the CSK is important in the process /...

the process of spatial segregation and so it is worthwhile determining the mechanism of mRNA association with the cytoskeleton.

In addition it has been observed that treating cells with cytochalasin D (CD), which releases mRNA but not proteins from the CSK, leads to differing amounts of message being released from the CSK depending upon the concentration of CD used to treat the cells. Any messages released lose their capacity to be translated, but those remaining bound are not affected (Fey et al, 1986). However the messages freed from the cytoskeleton still retain their capacity to be translated in vitro (Bag and Sarkar, 1976; Bag and Sells, 1979).

It is also worthwhile noting that in reticulocytes where considerable membrane-bound translation occurs, treatment with CD leads to little or no change in protein synthesis (Fey et al, 1986). This suggests that the change in protein synthesis rates observed is due to the messages becoming detached from the CSK.

Hence the mode of attachment of mRNAs to the CSK is worthwhile studying as it is vital in retaining the capacity for translation of messages, as well as probably having a role in spatial segregation.

In addition to terminating in vivo translation and dissociating the mRNA from the CSK, CD treatment also removes the ribosome from the CSK. Since the CD only affects the binding of mRNA to the CSK, and not proteins to the CSK, this adds weight to the theory that polysomes are bound to the CSK by their mRNA components (Cervera et al, 1981).

Before investigating the mechanism involved in protein-mRNA interaction /...

interaction, it is worthwhile considering the possibility that the association may be of a heterologous nature (Zambetti et al, 1990).

It has been shown that a protein homologous to the cap-binding proteins co-localizes with elements of the CSK (Zumbe et al, 1982). However co-localization may only be a reflection of secondary binding of the protein to the CSK via a common linkage to the mRNA. Better evidence exists that the protein component of the polysome plays a role in the binding of polysomes to the CSK (Howe and Hershey, 1984).

Returning to the idea of messages playing a role in polysome binding to the CSK, it would be interesting to examine which areas of the message actually are associated with the CSK. Setyono and Greenberg (1981) addressed this question by use of UV cross-linking of mRNA and proteins.

mRNA was passed over oligo (dT) cellulose to isolate only those proteins which bind to polyA$^+$ RNA. In the polyA region of messages a 78kDa protein was found bound. In addition a number of other proteins were found bound to the non-polyadenylated region of the polyA$^+$ RNA. Hence it is clear that RNA-protein interactions do occur, but this does not actually prove that mRNA binds to the CSK via the RNA component of the polysome. The 78kDa protein described above is found in most eukaryotes and the binding of the protein to the mRNA is very stable even at high salt concentrations (Jackson and Standart, 1990). However in order that it be determined if this protein is essential for protein-RNA interactions, it is advisable to study cell lines which favour translation of messages on membrane-bound polysomes, rather than free polysomes. One such cell is the rabbit /...

the rabbit reticulocyte, where the 78kDa protein is absent (Van Venrooij et al, 1977). Hence it seems that the 78kDa protein is not necessary for protein synthesis if the cell does not utilize free polysome translation.

By a process of deduction this suggests that the 78kDa protein has a role to play in the association of mRNA, and so polysomes, to the CSK. Furthermore, since the this protein specifically associates with the polyA region of the mRNA the polyA region is probably important in attaching the message to the CSK.

In the species Astemia salina a 38kDa protein associates with the polyA region of mRNAs. If the 38kDa protein is phosphorylated messages may be translated, but in the dephosphorylated state the mRNA is unable to be translated (de Herdt et al, 1984). This further enforces the critical role of polyadenylation in RNA translatability.

During nuclease digestion nucleotide sequences may be protected by interaction with proteins rendering them unavailable for digestion. By use of such treatment it was estimated that as much as 25% of an mRNA molecule is protected by proteins (Dubochet et al, 1973). By studying the nuclease resistant components of globin mRNA it was found that nearly all protein protection was afforded outwith the coding region. The only exception to this was a short stretch of nucleotides near the translation initiation site (Chae and Patton, 1984; Patton and Chae, 1983). Hence the actual protein encoding region of globin messages plays little part in binding messages to proteins.

Having discussed the roles of both mRNA and proteins in polysomes with regard to CSK binding, it would be interesting to discuss the cytoskeletal component involved in polysome binding.

Polysomes /...

Polysomes do not bind to the cytoskeleton via the intermediate filaments (Nielsen et al, 1983). However this is not particularly surprising for oligodendrocytes which lack all known intermediate filaments, but still have messages translated on free polysomes.

Secondly, mRNA binding to the cytoskeleton survives detergent extraction, even under conditions which disrupt microtubules. This suggests that microtubules are not responsible for anchoring messages to the CSK in HeLa cells at least (Lenk et al, 1977). Hence by a process of default it appears that the microfilaments are the prime candidates for the role of RNA association with the CSK. However positive proof that messages associate with actin is also available, as the association does not survive detergent extraction of cells which have been pre-incubated with the drug cytochalasin B, which disrupts microfilaments (Lenk et al, 1977; Isaacs and Fulton, 1987).

It is wrong however to view the components of the cytoskeleton as individual entities, due to their considerable interaction with each other. Hence Nielsen et al (1983) proposes that mRNA and ribosomes appear to associate with microfilaments directly or with a structure whose cytoskeletal attachment is dependant upon the integrity of the microfilaments.

The importance of other components of the CSK cannot be underestimated. For example microtubules and the endoplasmic reticulum are known to co-localize (Terasaki et al, 1986), and the microtubules are needed to recycle membrane-bound ribosomes (Walker and Whitfield, 1985). Therefore no single component of the cytoskeleton can be regarded as being the sole source of CSK-mRNA interactions.

Further /...

Further investigation of the results of the UV crosslinking suggest that a number of proteins, with molecular weights ranging from 52 to 98 kDa associate with the non-polyadenylated region of mRNA (Setyono and Greenberg, 1981; Larson and Sells 1987).

Having shown an association betwen the cytoskeleton and polysomes it is interesting to study the reason for this occurrence and to contemplate the implications of the association. Polysomes binding to the cytoskeleton may reflect a need for the message to be near appropriate initiation factors (Howe and Hershey, 1984), or it may reflect a means of helping the message to remain near the site where the eventual protein product will be involved in the cell at large (Capco and Jackle, 1982; Fulton and Wan, 1983). In truth the association probably has a role to play in both of these functions.

By using ^{35}S-methionine and allowing cells to undergo translation for ten minutes, the site of synthesis of nascent polypeptides can be investigated. By autoradiography it is clear that there are radioactive proteins found in the cytoskeleton even after such a short incubation period (Fulton et al, 1980). Hence protein synthesis is near the site of cytoskeletal incorporation and this incorporation into the CSK must be quite rapid (Fulton et al, 1980).

A measurement of how rapidly the nascent proteins and polypeptides incorporate into the cytoskeleton may be obtained using puromycin treatment, which releases polyribosomes from the CSK. Hence it is possible to determine if nascent polypeptides are actually associated with the cytoskeleton prior to the completion of translation. If the nascent polypeptides are already /...

are already associated, then in theory they should still bind to the CSK after puromycin treatment. By studying initiation factors Howe and Hersley (1984) showed that these nascent polypeptides have the capacity to associate with the cytoskeleton prior to the completion of translation.

Hence it seems that the polysomes are attached to the CSK via the proteins. In addition it seems that the polysomes are associated with the microfilamentous, or microfilamentous-associated component of the cytoskeleton. The reason this association exists seems to be to ensure that messages are held in a particular area to ensure the message is translated there and in turn that, if required, it can be rapidly inserted into the CSK, even prior to translation being completed.

In conclusion the fact that polysomes and the cytoskeleton interact is well documented, and well studied. However the significance of this relative to the process of mRNA transportation within the cytoplasm is a much less well defined area. All that is known about the transportation of mRNAs is that it occurs throughout in association with the cytoskeleton and that the process is an energy-dependent one (Fey et al, 1986).

1.4.5. ROLE OF mRNA SEGREGATION

It is generally accepted that mRNA segregation is employed to target the message to an area where it can be translated, and the newly synthesized polypeptide incorporated into its required site immediately (Capco and Jackle, 1982; Fulton and Wan, 1983; Lawrence and Singer, 1986). In addition, if the eventual protein is to be incorporated into the CSK, this can be done while translation is proceeding (Fulton et al, 1980).

In addition /...

In addition segregation is also advantageous as one message can be translated more than once. Hence instead of transporting numerous new proteins around the cell, it seems more efficient to transport a smaller number of messages to obtain the same nett effect.

Probably one of the best examples of mRNA segregation is the study of actin and tubulin messages (Lawrence and Singer, 1986) where messages are found in a similar subcellular location to their corresponding proteins.

1.4.6. *in situ* LOCALIZATION OF MYELIN mRNAs

In addition to the research already dealt with as regards *in situ* hybridization in cells derived from myelin mutant mice (Section 1.2.3.), work has also been performed to study the subcellular localization of myelin messages in wild type animals.

It is well documented that the MBPs are translated on free polysomes, but that those of PLP are translated on membrane-bound polysomes (Colman *et al*, 1982). In addition it has been shown that CNP messages are associated with free polysomes (Gillespie *et al*, 1990b). Hence it would seem logical to assume that the messages encoding PLP will have a tendency to be located somewhere within the cell body of the oligodendrocyte, but little can be predicted about the localization of the MBP and CNP encoding messages, as free polysomes are expected to be available for translation throughout the cell.

To date a number of studies have been performed to localize various myelin messages by use of *in situ* hybridization (Trapp *et al*, 1987; Vogel *et al*, 1988; Jordan *et al*, 1989; Shiota *et al*, 1989).

With the exception of Shiota's work, all research was performed on brain sections. Shiota used both brain sections and cells grown in tissue culture. However the work presented in the four papers listed above give similar overviews of the sub-cellular distribution of the various myelin specific messages- with the exception of MAG, which has not been studied.

Using a cDNA probe specific for a particular message, *in situ* hybridization has suggested a differential localization of various messages : CNP mRNA is found within the cell body of oligodendrocytes (Trapp *et al*, 1988; Vogel *et al*, 1988); MBP messages are found primarily in the cell body, but to a lesser extent within the cell's processes (Trapp *et al*, 1987; Jordan *et al*, 1989; Shiota *et al*, 1989); and PLP mRNA is found within the cell body (Trapp *et al*, 1987; Jordan *et al*, 1989; Shiota *et al*, 1989).

These results add weight to the argument that messages may be translated in the area ultimately targetted for the protein, within the oligodendrocyte. In the case of MBP, this protein is found in both the cell body and the cell's processes (Shiota *et al*, 1989; Wilson and Brophy, 1989), but PLP is primarily in the cell body (Shiota *et al*, 1989) as is true for CNP of mature oligodendrocytes (Wilson and Brophy, 1989).

In the work described above it is important to note that most of the previous work performed in this field was performed using brain sections rather than from oligodendrocytes grown in tissue culture. Hence the results presented later in this thesis can be compared with that already published for brain sections, and so give some indication of the degree of difference between cells obtained by two alternative routes.

1.5. AIMS OF THIS PROJECT

The primary aim of this project was to investigate the sub-cellular distribution of a number of messages encoding proteins found in the myelin of oligodendrocytes.

In the first instance it was thought better to use cDNA probes as a means of performing *in situ* hybridization, and an anti-actin clone was obtained from Dr. D. P. Leader (University of Glasgow). However it was decided at an early stage in the project to use oligonucleotide probes. Such probes can only be used where the DNA sequence of the intended target for probing is known. In the case of myelin proteins this is well known and a number of such DNA sequences have been published. Since oligonucleotides may be utilised it is worth-while dwelling on their advantages as opposed to the cDNA probes. Primarily they present an opportunity to study the various members of a gene family, as well as the various different differential splicing products found from a single gene. In addition the use of such probes does not rely on either construction of a DNA library, or obtaining cDNAs from other labs. Furthermore once obtained from a synthesizer there is no need for labour consuming work such as plasmid isolation and purification on a caesium chloride gradient.

Together with the work dealing with the sub-cellular distribution of the proteins in oligodendrocytes, which was being performed at the same time in this lab (Wilson and Brophy, 1989), it was hoped to compare the mRNA and protein distribution of each gene product in turn.

It was also decided to perform studies using cells grown *in vitro*, rather than brain sections. It was felt that this would /...

would make it easier to identify any messages found in the more peripheral areas of the oligodendrocyte. Nothing could be predicted as regards the likelihood of finding messages exclusively in the peripheral areas of the cell, but it is worthwhile noting that Lawrence and Singer (1986) reported the occurrence of actin messages in the peripheral areas of fibroblasts. Hence it was felt that using cultured oligodendrocytes had one particularly strong advantage over the use of brain sections.

The distribution of each message between the cytoskeletal and non-cytoskeletal areas was also to be investigated. This was to be initially studied using RNA which had been extracted from cells, although the possibility of studying the distribution of cytoskeletally associated messages within cells was also planned.

CHAPTER TWO

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. CELL CULTURES

2.1.1. OLIGODENDROCYTE CULTURES

Due to the complex morphology of the oligodendrocyte it is probably more informative to use cells grown in tissue culture, rather than brain sections for *in situ* hybridization. This is based upon the assumption that cutting an oligodendrocyte will seldom yield a cut which retains a cross-section of the entire cell. For this reason messages which lie specifically in the processes of the cell will possibly just appear as areas of background staining, and only messages clumped in a high density in one particular area will be easily detected (e.g. those in the cell body).

In order that oligodendrocytes may be grown in tissue culture it is essential to assume that the technique being used provides as true a reflection of oligodendrocyte development *in vivo* as possible. Obviously growth of oligodendrocytes in the absence of nerves denies them the chance to wrap around and myelinate their normal targets. Hence the processes of the oligodendrocytes may be artificially long, as they reach out from the cell body in an attempt to find nerves to ensheath. In addition there may be signals from other cell types absent from the cell's environment, creating an artificial situation. Another cell type which is thought to influence the development of oligodendrocytes is the type-1 astrocyte. This cell type is known to secrete growth factors which act on the O-2A cells which are the progenitor of both the type-2 astrocyte and the oligodendrocyte (Armstrong *et al*, 1990). One such factor is platelet-derived growth factor (PDGF) which can cause proliferation /...

proliferation of the O-2A cells and deters differentiation. PDGF is also thought to be produced by endothelial cells or microglia and exists as a dimer of 2 sub-units. These two sub-units can come in one of two forms A or B and so PDGF can be found as AA, AB or BB. Of the three forms present AA and AB appear to be more potent mitogens than BB. It is therefore interesting to note that the relative abundance of the A sub-unit tends to increase around E17 - E19 in embryonic rodents, which is the time at which oligodendrocytes begin to appear. However even in the absence of these factors the advantages of using cells grown in tissue culture can be considerable due to the complex morphology of these cells.

Numerous oligodendrocyte culture techniques are available, and many are based upon that of McCarthy and de Vellis (1980). This protocol was followed subject to the variations described in Wilson and Brophy (1989).

All cell manipulations were carried out under sterile conditions, and all instruments, equipment and solutions used were either autoclaved, filter sterilised or bought sterilely pre-packed. Dulbecco's Modification of Eagle's Medium (DME), fetal calf serum (FCS), gentamycin, glutamine and Hank's Balanced Salt Solution (HBSS)- without Mg^{2+} or Ca^{2+} were from Flow Labs. Insulin (I-5500), poly-D-lysine (P-7886), Sodium selenite (S-1382), transferrin (T-2252) and triiodothyronine (T-6397) were all from Sigma Chemical Company. Costar culture plastics and 10mm Chance glass coverslips (chromic acid cleaned) were used.

Until recently oligodendrocytes were though to be mitotically inert, which is still the view held for cells grown in tissue culture. /...

culture. In addition it is believed that the oligodendrocyte-type-2-astrocyte (O-2A) precursor cell is restricted in the number of divisions it can undergo before differentiating, if neurons are absent (Temple and Raff, 1986). Here to obtain a culture of oligodendrocytes primary brain cell cultures were set up, and oligodendrocytes enriched from the mixed primary culture. The mixed cultures were used on the basis of the observation that for an oligodendrocyte to obtain something like its in vivo morphology, under in vitro conditions, it firstly requires signals from astrocytes. Hence it is essential to delay the time of differentiation where possible (Bhat et al, 1981).

Primary glial cultures were from the cerebral hemispheres of 3-day old Wistar rat pups. Upon decapitation the heads were swabbed in 70% ethanol for a few minutes. The cranium was removed and the hemispheres extracted under sterile conditions. The cerebral hemispheres were transferred to chilled (4°C) HBSS (without Mg^{2+} or Ca^{2+}) and the meninges removed using sterile forceps. The hemispheres were then broken up firstly by chopping with dissecting scissors and the by trituration performed in a sterile pipette. This resulted in clumps of cells which required further breaking up before they could be used for tissue culture.

Clumps of cells were forced through 136μm nybolt mesh, followed by twice through 30μm mesh in the presence of chilled HBSS (without Mg^{2+} or Ca^{2+}). This created a single cell suspension. Cells were pelleted by spinning for 10 minutes at 200g and the resulting pellet resuspended in DME (containing 4mM glutamine) supplemented as follows:

	Final concentration	Stock
FCS	5%	100%
Gentamycin	50µg/ml	50mg/ml
Insulin	5µg/ml	500µg/ml
Sodium selenite	15nM	15µM
Transferrin	25µg/ml	2.5mg/ml
Triiodothryonine	15nM	15µM

(McCarthy and de Vellis, 1980; Wilson and Brophy, 1989). Insulin stock solution was stored at 4°C, and all other stocks were kept at -20°C.

For each pair of hemispheres, cells were resuspended in 10ml of DME (plus supplements). After resuspension the number of viable cells was typically around 3.75×10^7 per pair of hemispheres as determined by counting cells which exclude trypan blue. In addition it was found that by keeping centrifugation times to 10 minutes and only removing the major meninges that 48% of cells were still excluding trypan blue just prior to being seeded out. Longer centrifugation times, or spending longer removing the meninges often had a serious effect on cell viability, and could, over a period of only 15 extra minutes spent on the whole extraction procedure, lead to viability being lower than 20%.

Cells suspended in DME (plus supplements) were seeded at 5×10^5 viable cells/cm² on poly-D-lysine coated 75cm² plastic flasks. Flasks were pre-coated with 0.1mg/ml poly-D-lysine for 10 minutes, followed by a wash in ddH_2O prior to use. Cells were incubated in a humidified incubator at 37°C with a 4-6% CO_2 atmosphere.

Primary cultures were grown for 7 days in plastic flasks, having /...

having one media change after 3 days. Flasks were then gently tapped by hand and the media aspirated off. Fresh DME was added and again the base of the flask was gently tapped against the palm of the hand. This media was aspirated off and generally contained loosely attached cells and a few macrophages. HBSS (without Mg^{2+} or Ca^{2+}) was added to the flasks and the tapping procedure repeated. This media was also discarded and replaced by fresh HBSS (without Mg^{2+} or Ca^{2+}). This time the base of each flask was hit sharply against the palm of the hand and at once aspirated. This media was retained. The sharp strike process was repeated and the two fractions were pooled. These fractions, which contained a number of the O-2A progenitor cells were pelleted and resuspended in DME plus supplements (as above but using 1% FCS instead of 5% - this promotes differentiation in the direcion of oligodendrocytes [Temple and Raff, 1986]), and seeded at 50,000 viable cells/cm² on poly-D-lysine coated glass coverslips in uncoated plastic dishes. Using these conditions the resulting cell population had previously been shown within our lab to consist of 50-70% oligodendrocytes after 3 days further growth and differentiation, by use of anti-galactocerebroside antibodies.

For the purpose of <u>in situ</u> hybridization there is little point in proving the oligodendrocyte content of any particular coverslip as it could possibly prove difficult to then use proven cells for <u>in situ</u> hybridization. Instead only coverslips with around 30% or more cells adopting classical oligodendrocyte morphology were used for studies. Quite clearly cell form is not a perfect way to select cells since not all oligodendrocytes adopt this morphology; but at least it assures that at least some oligodendrocytes are present on the coverslip.

2.1.2. FIBROBLAST CULTURES

Hank's Balanced Salt Solution (HBSS) without Mg^{2+} or Ca^{2+}, Eagle's Minimal Essential Medium (EMEM), trypsin, and fetal calf serum (FCS), were all from Flow Laboratories. Costar culture plastics and 10mm Chance glass coverslips (chromic acid cleaned) were used. The human fibroblasts were derived from skin biopsies by Dr. R. Wilson.

Cells were grown in EMEM/ 10% FCS at 37°C in a humidified incubator containing 4% CO_2 atmosphere. Once the fibroblast population has grown to confluency the cells may be passaged, either onto fresh plastics, or onto glass coverslips. Passaging the cells involved aspirating off the culture media and briefly rinsing the cells in HBSS (without Mg^{2+} or Ca^{2+}) plus 10mM EDTA. This was followed by adding fresh HBSS (without Mg^{2+} or Ca^{2+}) plus 10mM EDTA and supplemented to 0.05% with trypsin. Flasks were allowed to sit for 20 seconds and all but a small quantity of the media, sufficient to wet the cells, was removed. In this state the cells were allowed to incubate for 3 minutes, which was sufficiently long for them to have rounded up. The cells could be detached quite easily by sharply tapping the flasks. Detached cells were then pelleted (10 minutes at 200g) and seed onto the required substratum.

2.2. RNA SAMPLES

2.2.1. RNA EXTRACTON

Basically two methods of RNA extraction were used; one for Northern blots and one for dot blots. The two methods used are described below and were used in preference to methods such as LiCl extraction (Cathala et al, 1983) purely on the basis of their being more rapid.

For dot blots RNA was extracted by the method of F. Ramalho-Ortigao (personal communications) based upon Gough (1988) using the detergent NP-40 for RNA extraction. This method was followed subject to further modifications described below.

Around 1g of rat brain tissue from a 15 day old animal was sonicated in 1ml of a buffer comprising 10mM Tris.HCl (pH 7.8), 10mM Sodium acetate, 10mM vanadyl-ribonucleoside complex (VRC). Sonication was performed on ice using a probe which had been washed in 20% SDS and was carried out until the solution was homogeneous, which normally required 5 minutes. Sonication was used in preference to Dr. Ramalho-Ortigao's method of homogenization by grinding as a higher yield of RNA resulted.

To the homogenate 100μl of NP-40 was added and the mixture vortexed briefly. The addition of undiluted NP-40 was found to be a further improvement on the existing method which used 100μl of a 10% solution of NP-40. The vortexed solution was allowed to stand on ice for 60 seconds before the addition of 1ml of 40mM Tris.HCl (pH 7.8), 40mM EDTA, 700mM Sodium acetate, 2% SDS.

This solution was then extracted by addition of 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1). This was then followed by extraction with 2 chloroform washes to remove as much /...

as much protein as possible. The resulting aqueous phase was ethanol precipitated. The final RNA pellet was resuspended in sterile ddH_2O and its purity determined by the $O.D._{260/280}$ ratio. Normally samples extracted by this method had ratio values in excess of 1.9 and never less than 1.8. This compares with a ratio of 2.0 for pure RNA. However attempts to translate the RNA extracted by this method by *in vitro* translation were usually unsuccessful, which suggests that the RNA may be degraded to some extent.

Hence RNA extractions for Northern blots were as described by Biogenesis Limited (Bournemouth) in their booklet concerned with use of the RNAzol method. Briefly this involved using 100mg of homogenised rat brain tissue in 2ml of RNAzol. To this 200μl of Analar chloroform was added. This mixture was shaken vigorously for 15 seconds, then placed on ice for 15 minutes, followed by a 20 minute centrifugation at 12000g at 4°C. After centrifugation two distinct phases are visible. RNA lies in the upper, aqueous phase and DNA and proteins are found in the lower, organic phase and at the interphase. As an additional precaution an equal volume of chloroform was added to the withdrawn aqueous phase, to remove any unextracted proteins. The centrifugation was again repeated and this time the aqueous phase was accepted as being sufficiently cleaned up for alcohol precipitation to be performed.

The first stage in the precipitation was addition of an equal volume of isopropanol This was stored at -20°C for 45 minutes, followed by centrifugation as previously described. The resulting pellet was then washed twice with 75% ethanol and then dried.

The RNA pellet was then dissolved in sterile ddH_2O and its purity and concentration /...

and concentration determined using the O.D. $_{260/280}$ ratio. This ratio value was normally in excess of 1.8, but rarely reached the 1.9 value obtained using the NP-40 method. However the RNAzol extracted RNA normally translated *in vitro* and since translatability of RNA is the generally accepted criterion for it being intact, the RNA was assumed to be full-length and so could be size fractionated for Northern blotting.

2.2.2. CYTOSKELETAL AND SOLUBLE FRACTION PREPARATIONS

The buffer used to extract the non-cytoskeletal components (referred to as soluble components) was as described in Gillespie *et al* (1989). Here the basic criterion for cytoskeletal residue requires that all tubulin remains in the cytoskeletal fraction (i.e. the detergent insoluble fraction) and that much of the actin is also retained.
However PLP, which is an integral membrane protein, is almost absent from the cytoskeletal residue and is found in the soluble component. As illustrated in Gillespie *et al* (1989) and Wilson and Brophy (1989), this criterion is achieved in a satisfactory manner by use of Triton X-100 as the detergent in the protocol descibed below.
N-p-tosyl-L-lysyl-chloromethane (TLCK), PIPES and Hepes were from Sigma Chemical Company; Triton X-100 was from Bio-Rad Laboratories; the proteinase inhibitors leupeptin and antipain were from the Peptide Institute, Osaka, Japan. All other chemicals were from BDH and of the highest purity available.
A single cell suspension of 15 day old rat brain material was created by forcing tissue through sterile 136μm and 30μm nybolt mesh in the presence of HBSS (without Mg^{2+} or Ca^{2+}). The resulting /...

resulting cells were pelleted by a 5 minute centrifugation at 1000g. The pellet was resuspended in Buffer CMT (see below) and gently washed. The cells were again pelleted and the resultant pellet dissolved in 1ml of pre-heated (30°C) Buffer CPT (see below) and allowed to incubate for 60 seconds at 30°C.

This solution was pelleted at 10600g at 20°C for 5 minutes. The resultant supernatant was removed and retained. The pellet was resuspended in 1ml of Buffer CMT and the 10600g centrifugation was repeated. The resultant supernatant was retained and pooled with the previous supernatant. This 2ml collective supernatant was termed the soluble fraction (SOL), and the remaining pellet termed the detergent insoluble, or cytoskeletal fraction (CSK).

Buffer CMT comprises 10mM PIPES (pH 6.9), 20% Glycerol 3.3 M $MgCl_2$, 50mM KCl, 2mM EGTA and 10μg/ml of each of leupeptin, Antipain, Pepstatin and TLCK.

Buffer CPT is the same as Buffer CMT with Triton X-100 added to a final concentration of 1.1%

In order that studies of distribution of messages be performed, the CSK and SOL fractions were treated as separate entities and RNA extractions were performed on each pool using the RNAzol method described in Section 2.2.1.

2.2.3. FIXATION OF CELLS

In the initial instance cells were fixed as described in Jirikowski et al (1988). Cells were fixed in freshly prepared 4% paraformaldehyde, 0.9% NaCl, PBS (pH 7.4) for 20 minutes at 37°C using fixative which had been pre-heated to 37° C. After fixing the cells they were washed briefly, twice in autoclaved PBS. These cells could then be stored at 4°C in PBS, 0.05% Sodium azide for several days before use. The fixation method described above was that routinely used in the labs of Drs Jirikowski and Ramalho-Ortigao.

It proved to be an acceptable method for the purpose of fixing cells for in situ/.....

(A)

(B)

Figure 11

Figure 11. A comparison of the MBP message distribution in cells fixed using different fixatives.

A) The cells have been fixed according to the protocol of Jirikowski *et al* (1988); 4% paraformaldehyde, 0.9% NaCl, PBS (pH7.4).

B) The cell has been fixed according to the protocol of Wilson and Brophy (1989); 4% paraformaldehyde, PBS (pH7.4), followed by quenching in 0.1M glycine, PBS.

From this figure it seems that as regards *in situ* hybridization very little difference is apparent between the fixatives in terms of MBP message distribution, which lies mainly in the cell body.

Size bar = 10μm

for *in situ* hybridization using anti-MBP oligonucleotide probes. However early attempts to use oligodendrocytes which had been fixed by this method for the purpose of immunocytochemistry proved unsuccessful (for conditions for immunocytochemistry see Section 2.4.4.). Two basic differences existed between the fixation method being used and that described in Wilson and Brophy (1989); a higher salt concentration in the fixative and the lack of a glycine incubation to quench any free aldehyde groups.

In an attempt to standardise the protocol for both *in situ* hybridization and immunocytochemistry - in order that the joint experiments may be permitted later - the fixation method used for immunocytochemistry was applied to the cells intended for *in situ* hybridization.

Cells were now fixed in 4% paraformaldehyde, PBS (pH 7.4) for 20 minutes at 37°C, followed by quenching of unreacted aldehydes with 0.1M glycine, PBS (pH 7.4) for 2x10 minutes and again washed twice for brief periods in autoclaved PBS. These cells could again be stored at 4°C in PBS, 0.05% Sodium azide for several days before use.

A comparison of the two *in situ* hybridization products using the two fixation methods is shown in Figure 11. It is quite clear that any difference present is minimal as regards the detection of MBP message distribution. Since the two methods seem to give identical results for *in situ* hybridization, there is little doubt that using the method which is also useful for immunocytochemistry is the more logical of the two.

Cells used for cytoskeletal extraction were treated with a brief wash in Buffer CMT followed by a room temperature extraction of three minutes in Buffer CPT (buffers described in 2.2.2.) /...

in 2.2.2.) as described in Wilson and Brophy (1989). It should be noted that there is a difference between the two extraction methods described in Wilson and Brophy (1989) and Gillespie et al (1989), however these methods had previously been developed to leave cytoskeletal residues in cells which had been extracted under different conditions; one from brain material and one from cells grown in vitro.

Having obtained a cytoskeletal residue, this part was fixed according to the modified protocol described above (suitable for immunocytochemistry). However, due to the cell being in a more fragile state, the extracted cells could only be stored at 4°C for a maximum of 2-3 days before use.

2.3. SELECTION OF HYBRIDIZATION CONDITIONS

2.3.1. CHOOSING AND LABELING PROBES

Three types of probes can be used for in situ hybridization; cDNA, riboprobes, and synthetic oligonucleotides. The first two are able to detect all members of a gene family, or a number of differential splicing products. Oligonucleotides also have this facility, provided the DNA sequence of all members of this group is known.

However when using probes to locate individual members of a gene family, or a specific differential splicing product, then oligonucleotides are essential. Due to the extensive DNA sequence information available for many myelin genes, and the number of genes which use differential splicing, the advantages of oligonucleotides are considerable.

Synthetic oligonucleotides were obtained from Oswel DNA Service (Department of Chemistry, University of Edinburgh) and were obtained HPLC purified. Radiochemicals were from Amersham and enzymes from Gibco/BRL Ltd. Formamide was from Fluka and Xylene Cyanol FF was from Bio-Rad.

These oligonucleotides can be labeled at either the 5' end, or the 3' end (Lewis et al, 1986). 3' labeling of DNA can be performed by use of terminal deoxynucleotidyl transferase (TdT), and 5' labeling used T4 polynucleotide kinase.

The method of phosphorylating oligonucleotides was as described in Lewis et al (1986), and briefly is as follows.

A reaction cocktail comprising 25mM $MgCl_2$, 25mM β-mercapto-ethanol, 8nM oligonucleotide, 3MBq/ml [γ-^{32}P]ATP (11.2MBq/nmol) 400 Units/ml T4 Polynucleotide kinase was used. A 12.5μl aliquot of this cocktail was reacted at 37°C for a minimum of 60 minutes. /...

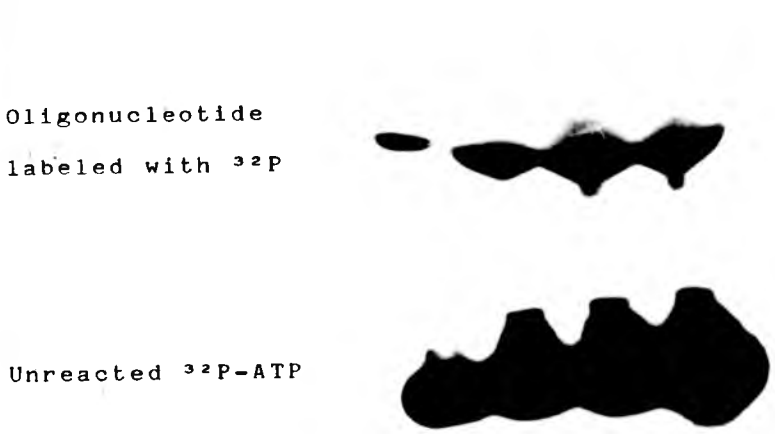


Figure 12.

Figure 12. An oligonucleotide labeled by use of T^4 polynucleotide kinase. The enzyme catalyses the removal of the ɣ-phosphate group from ATP and relocates it onto the 5' end of the oligonucleotide. The reactants have been applied to a 20% polyacrylamide, 6M urea gel and separated on the basis of size.

60 minutes. During this reaction the ɣ-phosphate group of the ATP is removed and transferred to the 5' of the oligonucleotide (Maniatis et al, 1982) as illustrated below:-

$$A\text{-}P\text{-}P\text{-}P^{32} + OH\text{-}C_p\text{-}C_p\text{-}C_p\cdots\cdots$$

$$\downarrow$$

$$A\text{-}P\text{-}P + {}^{32}P\text{-}C_p\text{-}C_p\text{-}C_p\cdots\cdots$$

All unincorporated ^{32}P may be removed using a single step G-50 Sephadex (Sigma Chemicals) column, swollen in ddH_2O, (Jirikowski et al, 1988). This reaction generally leads to considerable quantities of the ^{32}P being incorporated into the oligonucleotide.

By placing a small aliquot of the reaction mix in 99% deionised formamide, 0.3% Xylene Cyanol FF, 0.3% Bromophenol Blue, 0.37% EDTA (pH 7.0), it is possible to run the sample on a 20% polyacrylamide (acrylamide: bisacrylamide, 39:1) gel which contains 6M urea. This gel was run at a constant voltage of 400V for 2½ hours and the location of the oligonucleotide detected by autoradiography (Fig. 12).

The radioactively labeled oligonucleotide band lies between the Bromophenol Blue dye front and the Xylene Cyanol FF band (Lewis et al, 1986).

TdT sequentially adds deoxynucleotides to the 3' end of DNA molecules (Maniatis et al, 1982).

$$C_p\text{-}G_p\text{-}T + 2dATP$$

$$\downarrow$$

$$C_p\text{-}G_p\text{-}T_p\text{-}A_p + dATP + PPi$$

$$\downarrow$$

$$C_p\text{-}G_p\text{-}T_p\text{-}A_p\text{-}A_p + 2PPi$$

Figure 13

Figure 13. An oligonucleotide labeled by use of terminal transferase. The enzyme catalyses the addition of an additional nucleotide to the 3' end of the oligonucleotide. The resulting labeled probe has been run out on a 20% polyarylamide, 6M urea gel and separated on the basis of size.
The 4 lanes demonstrate use of different divalent cations in the enzyme reaction cocktail; (a)Mn^{2+}, (b) Mg^{2+}, (c) Co^{2+}, (d) Ca^{2+}.
The base line is evidence of a single nucleotide having been added, the next line is evidence of two nucleotides and so to the higher lines where as many as 20 or more nucleotides have been added. In addition it is quite clear that Ca^{2+} is not able to catalyse this enzymic reaction, as lane (d) shows no labeling.

Using this 3' addition method Lewis et al (1986) reported that up to 50 nucleotides may be added without affecting the hybridization specificity. Therefore it is in the best interest to select a tailing buffer which will generate maximum tail length.

Maniatis et al (1982) report that a divalent cation is essential for successful tailing, and list two examples. For this reason a number of different divalent cations were used to determine the optimal 3' tailing conditions (Fig 13) in the following buffer : 2mM $X^{++}Cl_2$, 0.5mM DTT, 32 Units/ml TdT 100mM Potassium Cacodylate, 8mM oligonucleotide, 4.5MBq/ml [α-^{32}P]dATP (11.2MBq/nmol).

This cocktail was incubated at 37°C for 45 minutes and the reaction was terminated by boiling for two minutes. Again using the treatment described above for kinase labeled probes, the oligonucleotides could be loaded onto and run on a gel and checked for successful incorporation of the ^{32}P.

From Fig. 14 it is quite clear that the most suitable divalent cation to use, in terms of maximal size is Co^{2+}.

As a quick check to determine the more efficient probe, in terms of sensitivity, a TdT labeled probe and a kinase labeled probe were used in a dot blot experiment, see Section 2.4.2. for methodology. From the resulting autoradiograph (Fig. 14) very little difference can be determined between the two methods. Hence kinase labeling was favoured on the basis of the enzyme being more stable over a period of time, and the fact that the time of incubation for the reaction is less critical.

Oligonucleotide probes were synthesized to be complementary to particular regions of the published sequences for rat myelin and cytoskeletal genes.

In theory /...

(A) Oligonucleotide probe
labeled at 3' terminus

(B) Oligonucleotide probe
labeled at 5' terminus

Figure 14

Figure 14. A comparison of two dot blots performed, one where the probe has been labeled at the 3' end and one where the probe has been labeled at the 5' end. On the evidence of the data presented here there is little difference in the signal generated by either the 3' labeled probe (A) or the 5' labeled probe (B).

In theory it should be possible to select any region of the published DNA sequence, however some method of selecting probes in a logical manner would be advisable.

Firstly the minimum size of a probe must be determined. This depends upon the organism's genome size, which in all mammals is typically in the region of $3x10^9$bp for a haploid genome. Assuming each nucleotide is present 25% of the time, then for a 16 base sequence to be present in the genome the probability is $(1/4)^{16}$, which is approximately $2.5x10^{-10}$. Hence the probability is that this 16 base sequence will be found only once in a haploid mammalian genome, and so in theory a 16mer is the minimum length of an oligonucleotide probe for mammals. However it is probably better to use a slightly longer probe to make it more likely that the sequence being targetted is uniquely present.

In addition to calculating the genome size it should be noted that very little of the entire genome is actually present as RNA. Probably only about 1% of the DNA in the genome is ever transcribed to give RNA, which makes the specificity of the oligonucleotide probe even greater.

Having decided upon the minimum length of the probe, it is now essential to decide which sequences to synthesize. This can be a fairly simple task if the study is focussing upon a particularly variable area of the message found in a gene family. However this is not always such a simple problem to tackle.

Since it was decided to investigate the possibility of using TdT as a means of labeling the probe this would lead to probes labeled with a string of a particular nucleotide at the 3' end.

If the first /...

If the first few nucleotides in this string are actually complementary to the next part of the message then this generates a longer probe and in turn would possibly raise the specificity.

For example the anti-actin probe TGG GGT GTT GAA GGT CTC 3' is an 18mer directed against nucleotides 373-390. This part of the actin message reads 5' gag acc ttc aac acc cct 3'. It is interesting to note that just 5' of this sequence there is a run of T_5 and so the terminal transferase labeled probe which reads 5' TGG GGT GTT GAA GGT CTC AAA AA can use the penta-A portion of the oligo-A tail as part of the hybridization recognition process.

The use of such a tail could be considered for labeling with dATP, dCTP or dGTP, but generating an oligo-T tail is not acceptable as this could lead to hybridization to the polyA tails of mRNAs, and lead to false positive signals.

Hence unless there is a specific reason for choosing a sequence to probe against, probes were generated against regions immediately 3' of a run of a particular nucleotide.

2.3.2. URIDINE LABELING

Enriched oligodendrocyte cultures (Section 2.1.1.) were used to study total RNA distribution within cells. The idea for this work came from Davis et al (1987), who worked with hippocampal neurons.

Cells were incubated in media used for enriched oligodendrocytes which was supplemented with 370kBq/ml 5,6^{3}H uridine (Amersham) for one hour. Cells were then given three brief (30 seconds) rinses in unlabeled media, and either fixed immediately in 4% paraformaldehyde /...

paraformaldehyde, PBS or grown in media containing 10μM unlabeled uridine (Sigma U-3750) for varying lengths of time (e.g. 2, 4, or 6 hours) before fixing.

All labeled cells were stained using immunofluorescence, to prove that they were oligodendrocytes. These cells were then dipped in Ilford K5 emulsion (Section 2.4.3.).

It was hoped that any labeled RNA would be found initially in the cell body, and as time progressed some would reach the more distal areas of the cell.

Initial attempts to label cells proved unsuccessful due to unacceptably high levels of background labeling. In an attempt to reduce this background, cells were initially incubated in 1μM unlabeled uridine prior to use of 5,6^{3}H uridine. This was done to reduce non-specific attachment of radioactive material to the substratum.

Use of the non-radioactive uridine as a blocking agent proved successful and the results generated by this technique are presented in Section 3.1.

2.4. HYBRIDIZATION CONDITIONS

2.4.1. NORTHERN BLOTS

Although Northern blotting is a well documented procedure, it can often prove a difficult technique to implement when using oligonucleotides as probes (Meinkoth and Wahl, 1984). A number of problems were encountered while trying to develop the Northern blotting technique for this particular work and development of the technique is discussed in detail in Section 3.3.1. However detailed below is the final protocol adopted which was used in the Northern blots described in Section 3.3.2. RNA gels were run as described by Maniatis et al (1982). Gels consisted of 1% agarose (Sigma A-6877) in a MOPS (Morpholinopropane-sulphonic acid) based buffer, in the presence of formaldehyde.

10x MOPS was defined as 400mM MOPS (pH 7.0), 160mM Sodium acetate, 10mM EDTA and could be made up in bulk quantities and stored at 4°C in the dark until required. Agarose was dissolved in ddH_2O (74ml per 1g of agarose), 10ml of 10x MOPS was added and finally 16ml of formaldehyde completed the gel mix. It should be noted that formaldehyde was stored as a 12.3M stock and that it must have a pH in excess of 4.0 before it may be used in RNA gels.

The running buffer used was 1x MOPS and the loading buffer was made as follows:

	Final concentration	Stock
RNA	4μg/lane	-
MOPS buffer	1x	10x
Formaldehyde	2.2M	12.3M
Formamide	50%	100%
Blue Juice	1x	10x

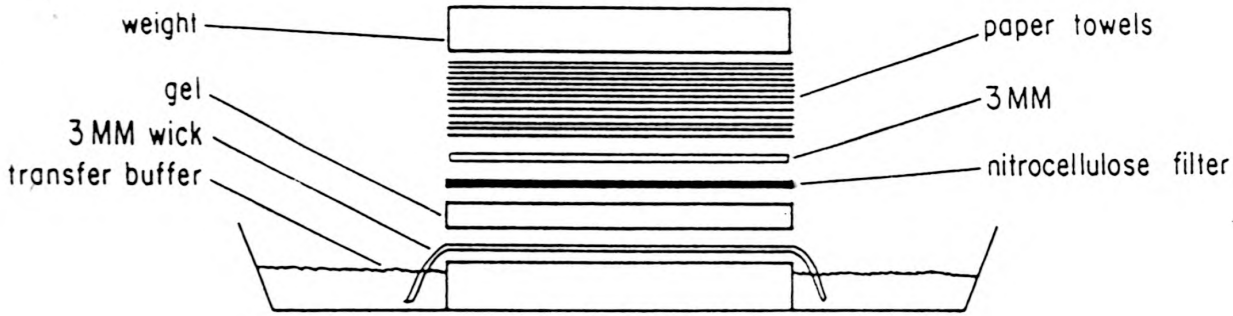

weight
gel
3 MM wick
transfer buffer
paper towels
3 MM
nitrocellulose filter

Figure 15

Figure 15. The transfer apparatus used to transfer RNA from agarose gels to nitrocellulose.

Before adding the Blue Juice, the buffer should be incubated at 55°C for 15 minutes, then quenched on ice. Formamide should have a neutral pH, which was ensured by passing formamide over a mixed-bed resin (Bio-Rad AG 501-X8). 10x Blue Juice consists of 50% Glycerol, 10mM Sodium phosphate (pH 7.0) and 0.4% (w/v) Bromophenol Blue.

MOPS used was from Sigma (M-1254) and Formamide from Fluka (47670). Gels were run at a constant 120V until the Bromophenol Blue dye front had migrated approximately 80% of the gel's length. The RNA was transferred from the gel to nitrocellulose (0.1μm pores) using the apparatus shown in Figure 15.

The transfer was carried out overnight and the extracted gel was stained with o.1μg/ml ethidium bromide to ensure no RNA remained in the gel. As a control segment of the gel which had not been transferred was stained, in accordance with Hames and Higgins (1985). Gels were not run in the presence of ethidium bromide on both safety grounds and also in the light of reports that intercalation of ethidium bromide into RNA can adversely affect the results of Northern blots.

After transfer the nitrocellulose was baked for two hours in a vacuum oven at 80°C, followed by a pre-hybridization for a minimum of six hours in the following buffer:

	Stock	Final Concentration
Formamide	100%	50%
Denhardt's Solution	100x	5x
Sodium Phosphate (pH6.8)	1M	20mM
SDS	20%	0.1%
E. coli tRNA	4mg/ml	30μg/ml
SSC	20x	5x

Denhardt's solution is defined as being 2mg/ml BSA, 2mg/ml PVP and 2mg/ml Ficoll /...

and 2mg/ml Ficoll in the 100x solution.

The pre-hybridization buffer can be stored in the dark at 4°C and was pre-heated to 42°C before being dispensed into bags for hybridization. 500µl of buffer was added per cm^2 of nitrocellulose.

Hybridization was performed using a buffer with slight modifications to the buffer used for pre-hybridization; Denhardt's solution was used at 1x and dextran sulphate (Sigma Chemicals D-6001) was added to 10% (w/v). In addition probe was added at a radioactive concentration of 10^6cpm/ml and specific radioactivity of 10^5cpm/pmole. Hybridization was carried out overnight at 42°C.

After hybridization the filter was washed five times at 37°C for five minutes in 3xSSC, 0.1% SDS when the filter was checked for radioactivity remaining by scanning with a Geiger counter. If the whole filter was still radioactive, it was washed a further twice at 37°C in 1xSSC, 0.1% SDS for 30 minutes. Otherwise the filter was scanned by autoradiography. Once the filters which had been given washes at higher stringencies were sufficiently clear of background radioactive contamination they too were placed under X-ray film (Kodak). Films were normally exposed overnight at -20°C and if necessary upon development this exposure time was increased to 48 hours to enable all signals to be sufficiently dense as to make photography possible.

2.4.2. DOT BLOTS

Development of the dot blot technique is described in Section 3.2.2. However the method finally employed is described below. 1.0μg of RNA was applied to nitrocellulose and allowed to air-dry only if the total volume added was 1.0μl or less, otherwise the drying process was accelerated by use of a jet of warm air from a hair-drier. At any one time a maximum of 1.5μl was applied. If a larger volume was being added to produce the required weight of RNA then only after one aliquot was fully dried was another added.

Having applied 1.0μg of RNA and allowing it to dry, nitro-cellulose strips were incubated for 2 hours at 80°C in a vacuum oven. Prehybridization was performed for 2 hours at 37°C in 7% SDS, 120μg/ml tRNA, 5xSSPE. After this 10 pmoles of radio-active probe per 1ml of pre-hybridization buffer was added to the prehybridization cocktail. The probe was labeled to 10cpm/pmole and was hybridized for 3 hours at 37°C.

Two washes of 2 minutes were carried out at 37°C in 2xSSPE, 0.1% SDS followed by a 2 minute wash at 55°C in 2xSSPE, 0.1% SDS. The washed nitrocellulose filter was allowed to air dry before being put under film for autoradiography.

As a technical foot-note 20xSSPE is defined as 3.6M NaCl, 200mM Sodium phosphate (pH 7.7), 20mM EDTA.

The overall methodology employed is based upon that of F. Ramalho-Ortigao (personal communications) subject to the modifications described in Section 3.2.2. It is also worth noting that Dr. Ramalho-Ortigao prefers the use of such a high SDS concentration as it seems to reduce the level of non-specific background labeling.

2.4.3. in situ HYBRIDIZATION

Cells were fixed as described in Section 2.2.3. and stored at 4°C. Before performing any work on these cells they were given time to recover to room temperature. Cells were then rinsed several times in PBS to remove all traces of Sodium azide. Intact cells (i.e. those not extracted with the CSK buffer) were permeablised in 0.1% Triton X-100, PBS for 5 minutes followed by several washes in PBS (Wilson and Brophy, 1989). All solutions were prepared in DEPC treated water, and where possible, were autoclaved. This was carried out in an attempt to minimise RNA degradation. The only exception to the DEPC treatment was any solution which contained any Tris based buffer, due to interactions between the two chemicals. In this case solutions were autoclaved routinely.

Pre-hybridization was carried out using 50µl of pre-hybridization buffer (see below for recipe) per coverslip for a minimum of one hour at 37°C in a moist incubator.

Pre-hybridization buffer was prepared by adding 1g of dextran sulphate to 5 ml of autoclaved 10xSSPE, 20mM EDTA, 100mM Tris.HCl (pH 7.0) and dissolved at 37°C. To this 20µl of 4mg/ml *E. coli* tRNA was added and allowed to dissolve, followed by addition of 5ml of deionised formamide. This buffer may be stored at 4°C in the dark until required.

After pre-hybridization, 0.4pmoles of labeled probe was added directly to the pre-hybridization buffer and hybridization was performed for two hours in the 37°C moist incubator. After hybridization, coverslips were washed in 0.9% NaCl, PBS, 0.05% Sodium azide (pH 7.1) for 10x 5 minutes at room temperature, followed by a wash at 37°C for 5 minutes and a final /...

final 5 minute wash at room temperature.

Cells were dehydrated through 50%, 70%, 80%, 90%, 95%, 99% and finally 100% ethanol. Once the cells on a coverslip had been dehydrated coverslips were attached - cell side up - to microscope slides using nail polish and dipped in Ilford K5 photographic emulsion. The emulsion was pre-heated to 37°C, diluted with 2 volumes of ddH_2O and allowed to settle at 37°C for one hour prior to dipping. This hour ensure that air bubbles were absent from the emulsion. Slides were dipped in the emulsion and allowed to dry at room temperature for two hours under a Kodak No. 1 filter safe-light. When dipping it is important to ensure that the coverslip is coated evenly with a very thin coating of emulsion.

Dry slides were placed in a light-tight, air-tight box with Silica gel present to remove any residual moisture, and allowed to expose for around 14 hours. Slides were developed in 16% (w/v) Kodak D-19 developer for 2 minutes and fixed in 30% (w/v) Sodium thiosulphate (photography grade) for 5 minutes, followed by several washes in ddH_2O for 30 minutes. Several changes of water was found preferable to washing in running water as variation in tap pressure over a 30 minute period was often liable to produce jets of water sufficiently strong to strip areas of the emulsion away from the slide. After washing in water the cells were mounted in 0.1xPBS, 90% glycerol and examined by microscopy.

As controls, sense-oligonucleotides and pre-treatment with RNase A at 37°C were used to determine the degree of non-specific hybridization present.

2.4.4. in situ HYBRIDIZATION AND IMMUNOCYTOCHEMISTRY

Cells which had been fixed as described in Section 2.3.3. and stored at 4°C were brought to room temperature and rinsed in PBS several times to remove all Sodium azide. Intact cells (i.e. non-CSK extracted cells) were permeablised with 0.1% Triton X-100, PBS for 5 minutes, followed by several washes in PBS (Wilson and Brophy, 1989).

All solutions were prepared in DEPC treated ddH_2O (except solutions containing Tris based buffers). In addition all solutions, where possible, were autoclaved.

Non-specific antibody binding sites were blocked using an incubation in 0.2% gelatin, PBS for 45 minutes. All antibody incubations were performed for one hour at room temperature using antibodies diluted at the appropriate dilution factor in blocking buffer (for dilution factors see Section 2.4.7.). Incubations were followed by 3x15 minute washes in blocking buffer.

All reactions, including washes, were performed at room temperature and in the case of fluorescently labeled secondary antibodies, or Rhodamine-phalloidin, incubations were performed in the dark. During the final wash of the fluorescent antibody, it is possible to leave the cells washing in the dark at 4°C overnight.

After completing the immunocytochemistry cells were washed in several changes of PBS for 30 minutes at room temperature in the dark. After the final rinse cells were checked briefly for successful fluorescence. If fluorescence was present cells were fixed as described in Section 2.3.3. and then *in situ* hybridization was performed as described in Section 2.4.3.

2.4.5. in situ HYBRIDIZATION USING RNA PROBES

Radioactive probes were prepared in the lab of Prof. Griffiths (Glasgow University Vet School) and the *in situ* hybridization protocol used was that used in his lab, and is based upon that of Cox *et al* (1984).

Riboprobes were prepared as described in Hames and Higgins (1985) by using SP6 polymerase. Briefly this involves transcribing cDNA in the presence of radioactively labeled UTP. As the uridine is incorporated into the message being transcribed, so an RNA probe, labeled to a very high specific activity, is generated.

The reaction is also performed in the presence of non-radioactive forms of the remaining three triphosphate nucleotides in a 6mM Mg_2Cl, 10mM DTT, 40mM Tris.HCl (pH 7.5) buffer. The labeled probe may be obtained after phenol extraction and ethanol precipitation.

No pre-hybridization was performed and the hybridization buffer was as described below:

	Final Concentration	Stock
Formamide	50% (v/v)	100%
Dextran sulphate	10% (w/v)	50%
Denhardt's solution	1x	100x
Tris.HCl (pH8.0)	20mM	1M
NaCl	0.3M	5M
EDTA	5mM	0.1M
Sodium phosphate (pH8.0)	10mM	0.1M
E. coli tRNA	0.5mg/ml	10mg/ml
Probe	10^5cpm/μl	10^6cpm/μl
DTT	10mM	1M

Hybridization was performed overnight at 50°C in a slide box moisturised by a tissue soaked in 50% formamide, 5xSSC.
Cells were washed for 20 minutes at 65°C in 2xSSC, 50% formamide, 0.1M DTT. This was followed by 3x10 minute washes in 0.5M NaCl, 10mM Tris.HCl (pH 7.5), 5mM EDTA at 37°C. This detaches the non-hybridized RNA probe.
Single stranded RNA was broken down by adding RNase to a final concentration of 5μg/ml to the 37°C wash buffer and allowing to incubate for 30 minutes at 37°C. This was followed by a 15 minute wash at 37°C in the previous buffer, and a 20 minute wash in 2xSSC, 50% formamide 0.1M DTT at 65°C. The wash procedure was completed using two room temperature washes, one in 2xSSC, and one in 0.1xSSC.
These coverslips were attached to microscope slides using nail polish and were dipped as described in Section 2.4.3.

2.4.6. OLIGONUCLEOTIDE PROBES USED IN THIS WORK

The following oligonucleotide probes were used in this work and are listed below by their intended target sequence and target nucleotides within the message

Anti-MBP

CGC ACC CCT GTC ACC GCT - nucleotides 163-180

G ATC TTG GGT CCT CTG - nucleotides 51- 66 of exon III

Anti-CNP

CTT GTC CTT AGG TCC TGA - nucleotides 10-27

ATC TTG AAG GAA AGG GAA - nucleotides 40-57

Anti-PLP

TTG ATG TTG GCC TCT GTA ACC CCT CCC CTT - nucleotides 361-390

Anti-DM-20

GGT GAT GCC CAC AAA CGT TGC GCT CAG GCC - nucleotides 331-345 & 451-465

Anti-PLP/DM-20

GAA ATA GGT CTC AAT TAG CTT TTC TGT ACC - nucleotides 121-150

Anti-actin

ATA GCA CAG CTT CTC TTT - nucleotides 634-651

TGG GGT GTT GAA GGT CTC - nucleotides 372-387

Anti-tubulin

CCC ACC AAA GCT GTG G - nucleotides 522-537

AAC ATT CAG GGC AGC GAT - nucleotides 832-849

With the exception of the PLP and DM-20 specific oligonucleotides, two probes were synthesized for each message. However the PLP and DM-20 messages are also being detected twice by virtue of the general probe for the two messages in addition to the two individual probes. It should also be noted that the nomenclature used to denote /...

used to denote the anti DM-20 probe is based upon the sequence of the PLP message and if the numbering was based purely on the DM-20 message this would read nucleotides 331-360.

The sources used for the various DNA sequences from which the oligonucleotide probes were synthesized are as follows; MBP (Roach et al, 1983); CNP (Bernier et al, 1987); α-tubulin (Lemiscka and Sharp, 1982); β-actin (Nudel et al, 1983); DM-20 and PLP (Nave et al, 1987).

In addition two sense-oligonucleotides were used to show that sense-sequences do not hybridize to messages. These could really have been any two randomly chosen sequences but it was felt better to use one actin and one tubulin. These sense probes should be able to detect denatured DNA and so their inability to generate a signal is further illustration of the fact that all that is being detected is mRNA and that the signal is not due in any way to genomic DNA which has become denatured.

The sense probes used are as follows:

Sense-tubulin

CTC TCT GTC GAC TAC GG - nucleotides 568-584

Sense-actin

ACC ACC ACA GCT GAG AGG G - nucleotides 601-619.

2.4.7. ANTIBODY PROBES USED IN THIS WORK

The antibodies used in this work are as described in Wilson and Brophy (1989) and in summary are as follows:

PLP - gift from E. Carey, University of Sheffield

CNP - gift from D.R. Colman, Columbia University

MBP - gift from D.R. Colman

Tubulin - gift from J. Kilmartin, M.R.C. Cambridge

Rhodamine-phalloidin - Sigma Chemicals

All secondary antibodies were obtained from North East Biochemicals.

With the exception of the PLP antibody, which had a particularly low titre, all antibodies were used at a 1:100 dilution in blocking buffer. In the case of secondary antibodies which carried a fluorescent label - either rhodamine or fluoroscene - incubations were performed in the dark. The PLP antibody proved very dilute and was used at a 1:3 dilution.

The specificity of all these antibody probes is discussed in some detail in Wilson and Brophy (1989). However it is worthwhile noting here that all the antibodies detect only those proteins against which they were raised, as based on Western blotting.

CHAPTER THREE

RESULTS

3. RESULTS

3.1. URIDINE LABELING

3.1.1. DEVELOPMENT OF URIDINE LABELING TECHNIQUE

The technique described by Davis et al (1987) proved fairly successful when applied to oligodendrocytes, subject to a few minor changes. Using a uridine labeling technique provided a unique opportunity to illustrate that the fixation process being used does not significantly digest RNA.

Enriched oligodendrocyte cultures were obtained as described in Section 2.1.1. and fixed as described in Section 2.2.3. after incubation in 5,6 ^{3}H uridine (Amersham) - see Section 2.3.2. All labeling incubations were performed on coverslips which had cells growing on them. This reduced the volume of liquid required for the hour-long incubation in 5,6 ^{3}H uridine. However all incubations with labeled uridine were performed in 6-well plates containing a number of coverslips. This is explained as the period of 60 minutes being the maximum time that a coverslip at 37°C can be left without fear of significant volumes of buffer evaporating.

Once cells were ready they were fixed in paraformaldehyde (see Section 2.2.3.) and the coverslips were stuck onto microscope slides using clear nail polish. Slides were then dipped in Ilford K5 emulsion (Section 2.4.3.) and examined using bright field microscopy. Without exception all slides were covered with silver grains which were distributed in a highly non-specific manner (Fig. 16).

This non-specific distribution was thought to be a problem of the radioactive uridine binding to the substratum due to the gaps lying /...

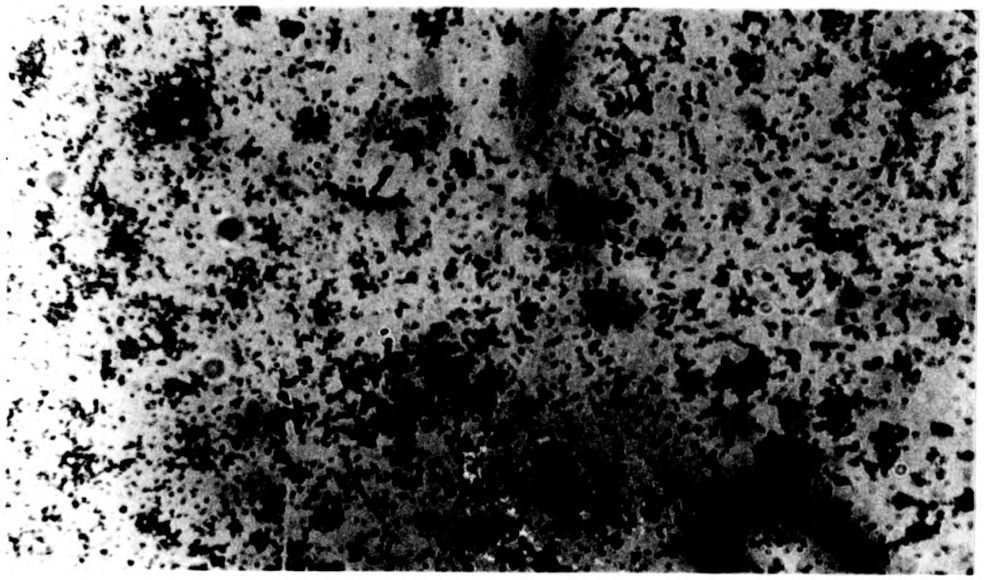


Figure 16

Figure 16. Cells grown in the presence of 5,6^{3}H uridine but without any pre-incubation in non-radioactive uridine. Quite clearly the level of background staining is unacceptably high. Hence the decision was taken to use a pre-incubation with non-radioactive uridine.

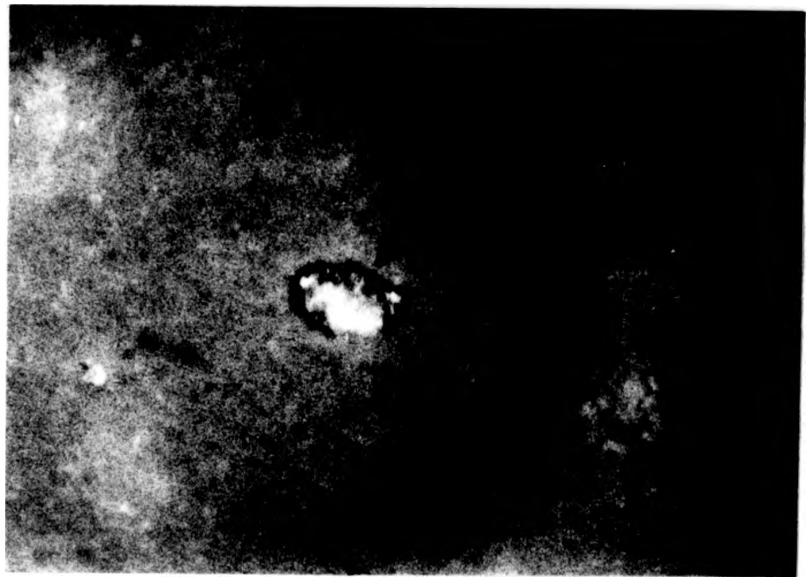
Sizer bar = 10µm

gaps lying between cells. In an attempt to overcome this problem the incubation with radioactive uridine was preceeded by a 1μM non-radioactive uridine incubation. This pre-incubation proved necessary as now the non-specific background became sufficiently reduced to be tolerable (Fig. 17-20).

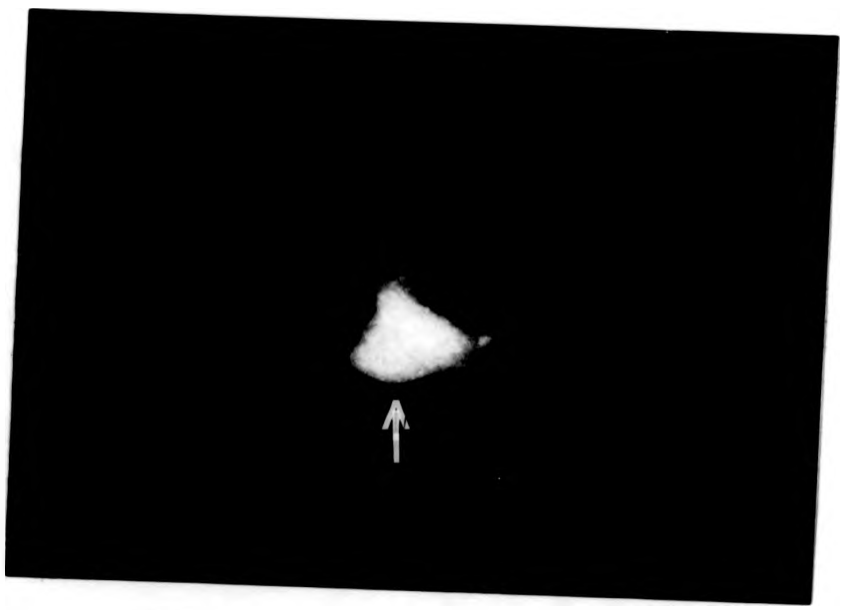
Since the signal detected by the emulsion revealed the distribution of nascent messages this also suggests that the fixation method being used is successfully fixing the RNA. This is an important point to verify as previously the fixation method being used here had only been tested on proteins (Wilson and Brophy, 1989).

The next important point to verify is that the cells may be probed using immunocytochemistry. This is very important in this work as it is the only way to prove that a particular cell being examined is an oligodendrocyte. In the case of a cell which has been allowed sufficient time for nascent messages to disperse through-out the entire cell, the cell's shape will be visible and give an indication of the cell being an oligo-dendrocyte. In addition in all cells phase contrast microscopy will often give an idea of the cell's type. However once dipped in emulsion there is often a problem using phase contrast microscopy with any success. In addition use of Nomarski polarization proved unsuccessful after dipping. Immunocytochemistry was successfully performed at the first attempt and even after dipping slides in emulsion the fluorescence could easily be detected through the emulsion (Figs.17,19&20). This successful fluorescence is also a highly important point to note as prior to this experiment it was not known if the emulsion would allow fluorescence to be detected, or if it would act as some form of barrier.

Hence /...



(A)



(B)

Figure 17

Figure 17. An oligodendrocyte grown in the presence of 5, 6 ^{3}H uridine for one hour and fixed at once after the incubation. The nascent messages are found in the cell body indicated by the arrow (A). The fact that the cell being investigated is an oligodendrocyte, is demonstrated by immuno-cytochemical means using an anti-PLP antibody, as indicated by the arrow (B).
The sizer bar = 10μm.

Figure 18

Figure 18. An oligodendrocyte as determined by cell morphology, fixed 6 hours after incubation with ^{3}H-uridine. The radioactive RNA is detected by autoradiography and bright field microscopy. The messages being detected lie throughout the entire cell.

The size bar = 10μm.

Hence this work has not only proved that the distribution of messages with time may be studied in oligodendrocytes, but also the following two points;

i) the fixation protocol is capable of fixing messages throughout the entire cell,

ii) fluorescence may be detected through the Ilford K5 emulsion.

This second point is particularly important in the joint in situ hybridization and immunocytochemistry work.

3.1.2. DISTRIBUTION OF NASCENT MESSAGES

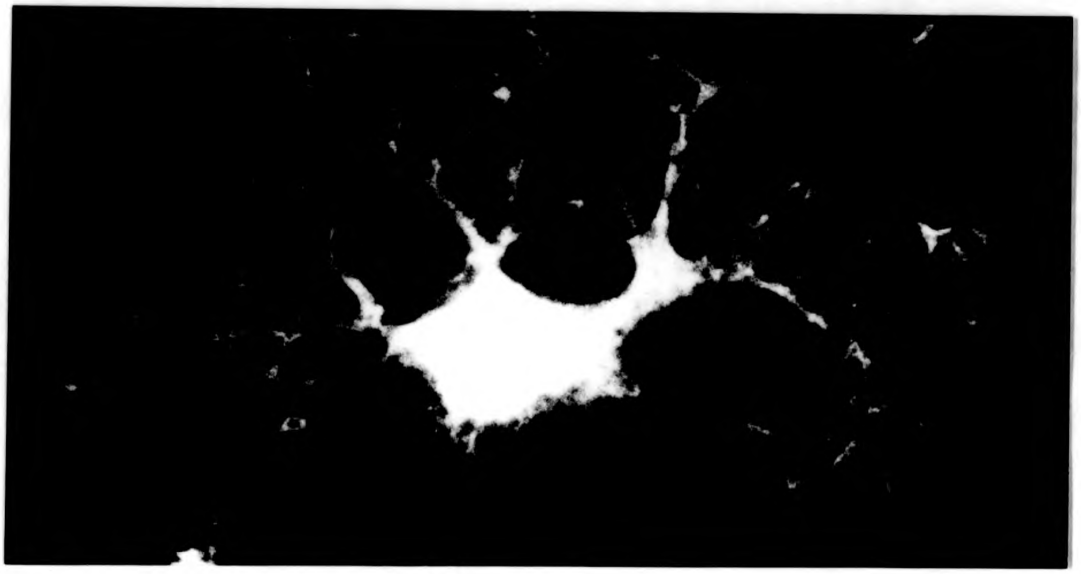
Distribution of nascent messages was studied by use of the techniques described in Section 2.3.2.

Figure 17 demonstrates the distribution of radioactively labeled messages immediately after the incubation with the 5,6 ^{3}H uridine. As might be expected the nascent RNA is found in the centre of the cell - the cell body.

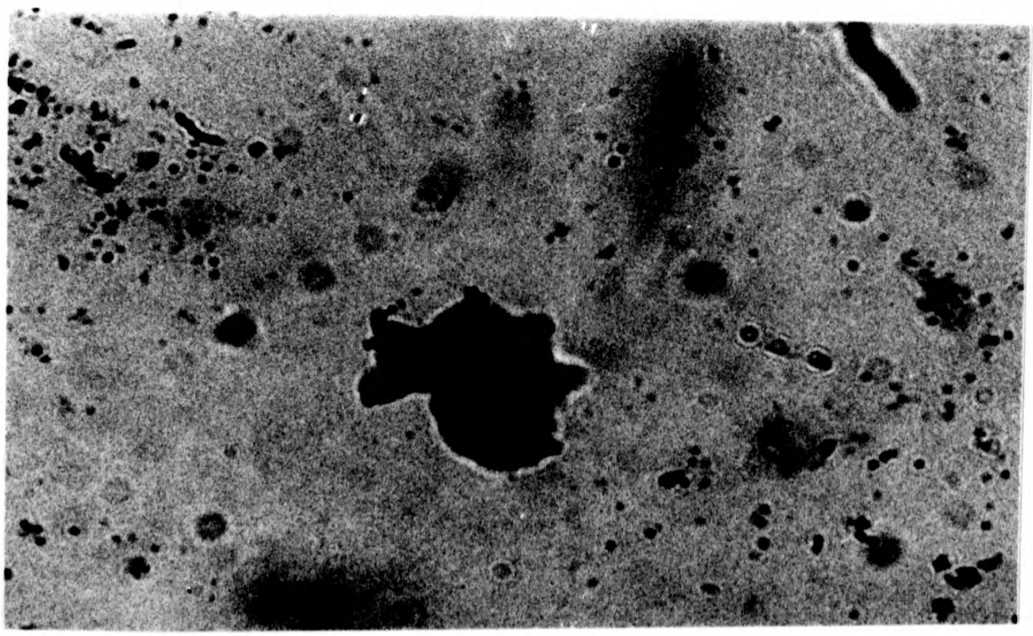
Figure 18 shows the distribution of radioactively labeled messages through time after being incubated in 10μM non-radioactive uridine. With longer times after the original incubation in 5,6 ^{3}H uridine, the radioactively labeled messages are found further from the nucleus, gradually reaching the most peripheral areas of the cell.

Figures 19 and 20 reflect the same distribution patterns seen in Figures 17 and 18. However the difference here is that the patterns are being studied using cells which have had their non-cytoskeletal components removed before fixation (see Section 2.2.3.).

In summary these figures demonstrate clearly what seems a logical /...



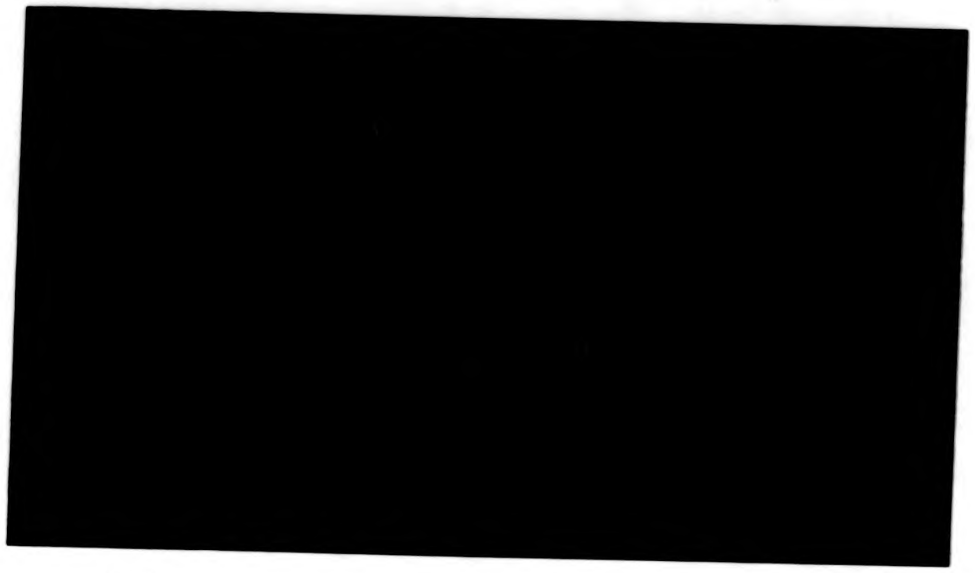
(A)



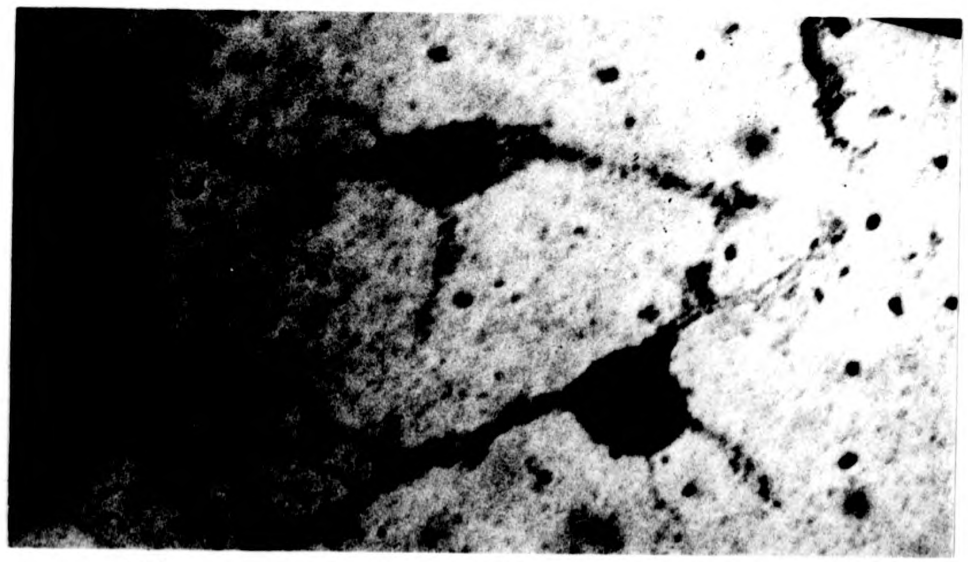
(B)

Figure 19

Figure 19. The CSK of an oligodendrocyte fixed immediately after incubation with ^{3}H-uridine. (A) The cell residue showing the MBP distribution as detected by indirect immunofluorescence. (B) The nascent RNA which associates with the CSK as detected by autoradiography and bright field microscopy. The size bar = 10μm.



(A)



(B)

Figure 20

Figure 20. The CSK of oligodendrocytes fixed 6 hours after incubation with ^{3}H-uridine. (A) The cell residues show the MBP distribution as detected by indirect immunofluorescence. (B) The distribution of recently transcribed RNA as detected by auto-radiography and viewed by bright field microscopy. The size bar = 10μm.

logical assumption; that messages are synthesizd in the centre of the cell, and only gradually are they transported to the extremities of the cells. However another point is also worth noting; the messages found in association with the cytoskeleton are found throughout the entire cell. This observation is in keeping with the theory that transportation of messages throughout the cell is done in association with the cytoskeleton. This of course is not the only reason for finding the messages in association with the cytoskeleton as some of the messages are not being transported, but instead have reached their targetted site in the cell, where they remain associated with the cytoskeleton.

The results generated in this section demonstrate the fact that RNA is found through out the cell. However they also show a much more important point:- that the fixation method being used is capable of generating RNA fixation. This point cannot be overemphasised as prior to this set of experiments the fixation method had only been used to demonstrate protein fixation in oligodendrocytes.

In addition these results demonstrate the fact that use of Ilford K5 emulsion does not prevent detection of fluorescent labels beneath the emulsion layer. In the light of the work presented later in this thesis it is most important to check that RNA fixation is achieved to a satisfactory level, and that there is a possibility of performing joint <u>in situ</u> hybridization and immunocytochemistry. The use of the duel techniques is discussed in greater detail at a later stage in this thesis, however at this point it is important to note that emulsion dipping does not prevent detection of fluorescent signals beneath the emulsion layer.

3.2. NORTHERN BLOTS

3.2.1. DEVELOPMENT OF THE NORTHERN BLOTTING TECHNIQUE

Use of RNA dot blots is a very useful technique for studies of the presence/absence of a specific message within a pool of total RNA. However before using any particular probe it is essential to check that it only detects the specific required message before being used on a dot blot.

In practice it is technically impossible to actually prove that any particular probe detects one message type, and only one message type. Instead the method of preference is to demonstrate that the probe hybridizes to messages of only one particular size, as determined by size fractionation on an agarose gel prior to transfer to a filter such as nitrocellulose or Hybond nylon.

Probing the size fractionated RNA does not show that only one species of RNA is being detected. Instead this technique of Northern blotting shows that only messages of a particular size are being detected. It is quite possible that two species of RNA of the same size are being detected simultaneously and appear as only one band on a gel. However in practice this is unlikely to occur and so Northern blotting is accepted as a means of demonstrating the specificity of any particular probe.

In general most techniques for Northern blotting have been developed for cDNA probes rather than oligonucleotide probes. However a number of such methods were used in an attempt to perform checks on the oligonucleotides used for work described in this thesis.

The most obvious method is to test first with the protocol being /...

being used for the dot blot research. The hybridization protocol described in Sections 3.3.2. and 2.4.2. was tested with the anti-actin oligonucleotide probe

TGG GGT GTT GAA GGT CTC

(nucleotides 637-654 of rat β-actin) and also with the anti-tubulin oligonucleotide probe

AAC ATT CAG GGC AGC GAT

(nucleotides 832-849 of rat α-tubulin). No band was detected on the autoradiograph of the Northern blot, even after a 48 hours exposure.

Quite clearly this protocol as it stands is unacceptable for the purpose of Northern blotting. However all subsequent blots using total brain RNA were tested with both the actin and the tubulin probe. Using these probes is logical as opposed to the probes for myelin specific messages when using total brain RNA, as all brain cells contain tubulin and actin messages, but not all cells in the brain contain myelin specific messages. Hence these probes, which had been successfully shown to hybridize to RNA on dot blots, were selected for further testing of various Northern blot techniques.

In all cases below the gels were run in a MOPS/formaldehyde gel and transferred onto 0.45μm pore nitrocellulose as described in Section 2.4.1. In addition, all hybridizations were performed by addition of 12 pmoles of oligonucleotide probe to the pre-hybridization buffer. Both hybridization and pre-hybridization were performed in a sealed freezer bag using 10ml of buffer, which gave a filter covering of 0.5ml per cm^2 of filter, and filters were rocked on a rocking platform in a heated incubator. After washing, filters were exposed to X-ray film for 48 hours before /...

before developing. All protocols were repeated twice for each probe before any protocol was dismissed.

It should be noted that in the work detailed below T_m for the anti-actin probe was defined as 56°C and T_m was defined as 54°C for the anti-tubulin probe. These values were obtained from the following equation:

$T_m = 4x(\text{number of G+C molecules}) + 2x(\text{number of A+T molecules})$

The next hybridization method used was that described in Maniatis et al (1982) and was performed at (T_m-5)°C using 6xSSC, 0.5% SDS, 5x Denhardt's solution, 30μg/ml sonicated salmon sperm DNA. Pre-hybridization was performed for 3 hours and hybridization was performed overnight.

The filter was washed in 2xSSC, 0.5% SDS for 5 minutes at room temperature, followed by another room temperature wash for 15 minutes in 2xSSC, 0.1% SDS. More stringent washes were performed for 2x1hour in 0.1xSSC, 0.5% SDS at (T_m-5)°C. Neither probe proved able to detect the intended target by use of this method.

An attempt was made to carry out the hybridization at a lower temperature by using 50% formamide instead of 0.5% SDS. This allowed the hybridization to be carried out at (T_m-25)°C. All other variables in the hybridization and pre-hybridization protocol were unchanged.

The wash procedure used was that previously described. However again only negative results were obtained using both the actin and the tubulin probes.

It was possible that the wash procedure being used above was too stringent and even complementary probes were being washed off. For this reason the original hybridization conditions were reverted /...

reverted to (i.e. 6xSSC, 0.5% SDS, 5x Denhardt's solution, 30μg/ml DNA at T_m-5). but washes were performed at higher salt concentrations, which decrease the stringency of the washes. In addition all washes were shortened to only 5 minutes, and performed at room temperature. The first wash was in 6xSSC, followed by 2xSSC, 0.1% SDS and 1xSSC, 0.1% SDS. Again no band was detected using either probe.

Such a low stringency of washing suggests that the problem is not actually in the washing process, but rather in the hybridization. Due to there being no evidence of high background on the autoradiographs it was decided to omit the Denhardt's solution from both the pre-hybridization and the hybridization buffers. This decision resulted in a hybridization buffer similar to that used by Murasuigi and Wallace (1984), so this protocol was followed as they described.

A 3 hour pre-hybridization was performed at (T_m-5)°C using 6xSSC, 0.1% SDS, 1mM EDTA, 30μg/ml sonicated salmon sperm DNA, followed by an overnight hybridization at (T_m-5)°C . Four 5 minute washes were performed at room temperature using 6xSSC followed by a 5 minute wash at (T_m-5)°C with 6xSSC. Again both probes proved unsuccessful under these conditions.

Since it had been proposed that the problem lay in the hybridization technique, rather than the washing, it was decided to use an alternative salt solution to replace the 6xSSC. One such solution is mentioned in Meinkoth and Wahl (1984) and this was the next method used.

The buffer used for hybridization comprised 90mM Tris.HCl (pH 7.4), 900mM NaCl, 6mM EDTA, 30μg/ml sonicated salmon sperm DNA, 0.1% SDS, and hybridization was performed at (T_m-5)°C. Two 5 minute washes were performed at room temperature, using 1xSSC /...

1xSSC, 0.1% SDS, followed by two 15 minute washes using 0.1xSSC, 0.1% SDS at (T_m-20)°C. No band was detected by the tubulin probe on the two occasions tested. However a single very faint band was observed on the autoradiograph. Unfortunately the band was so faint that it proved impossible to photograph. However even such a faint band was treated as a potentially positive sign, and the method was repeated a further three times before it was rejected as being unreliable.

Since there appeared to be a problem in the hybridization method it was decided to check that the RNA had transferred from the gel to the nitrocellulose. After transferring the RNA, the gel was checked on the transilluminator and no RNA could be found on the gel. Instead fluorescence could be detected on the nitrocellulose, suggesting that successful transfer had taken place. However one important point was observed; the ribosomal RNA no longer appeared as sharp bands, as was the case in the gel, but instead was a smear.

This suggests that the RNA may not be presented in a form suitable for hybridization and indeed the RNA may lie as a smear rather than the intended sharp bands. Since most Northern blot techniques have been described for cDNA probes it was decided to investigate the form of RNA by use of a cDNA probe. The probe used was an MBP cDNA which was a gift from Prof. I.R.Griffiths (Glasgow Univ. Vet. School). This was labeled by random priming using a random priming kit from BRL/Gibco.

A Northern blot was performed according to the protocol routinely used in Prof. Griffith's lab. The pre-hybridization buffer comprised 50% formamide, 5xSSC, 5x Denhardt's solution, 20mM Sodium Phosphate (pH 6.8), 0.1% SDS, 30μg/ml *E. coli* tRNA and prehybridization /...

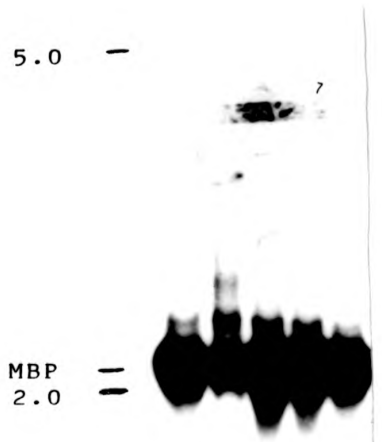


Figure 21

Figure 21. A Northern blot used to detect MBP encoding messages, by use of a cDNA probe. The band of interest is the lower band, the upper band being an area of sufficiently high complementarity to the MBP message as to allow it to be detected. The markers are 2.0kb and 5.0kb.

and pre-hybridization was performed at 42°C for 6 hours. Hybridization was performed overnight at 42°C in a buffer similar to that used for pre-hybridization, the difference being the addition of Dextran sulphate to 10% (w/v) and the Denhardt's solution reduced to 1x.

After hybridization the filter was washed five times for 5 minutes each at 55°C in 3xSSC, 0.1% SDS. The filter was then scanned with a Geiger counter. If the whole filter was still radioactive, it was washed a further two times in 1xSSC, 0.1% SDS for 30 minutes at 55°C. Otherwise the filter was placed under X-ray film. Once the filter was washed at higher stringency it was sufficiently clear to put under film. Again this method proved unsuccessful for detection of the MBP messages and since it had been routinely used in Prof. Griffith's lab, this presents additional evidence that the form of the RNA on the filter is not optimal.

In an attempt to overcome this difficulty the membrane was changed to a 0.1μm nitrocellulose filter. It was hoped that the smaller pore size may in some way help present the RNA in a form more readily accessible for hybridization. The probing technique with the MBP cDNA was repeated using the 0.1μm nitrocellulose and the result may be seen in Figure 21.

The technique which had proved successful using the cDNA probe was repeated using the anti-MBP oligonucleotide:

CGC ACC CCT GTC ACC GCT

which is complementary to nucleotides 163-180 of the rat MBP message. Hybridization was performed at (T_m-25)°C instead of 55°C (i.e. 37°C) due to the presence of 50% formamide in the hybridization buffer. However washes were performed at 55°C.

After /...

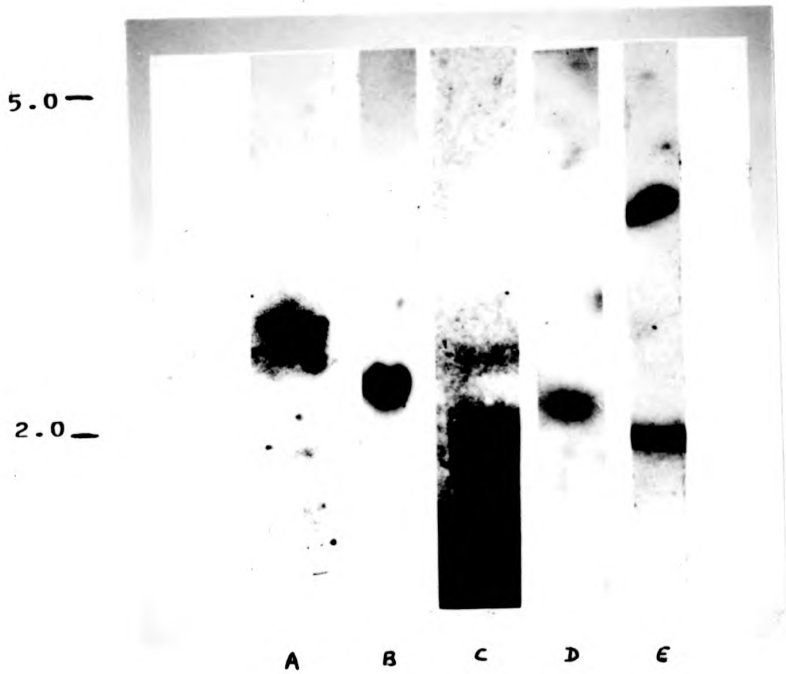


Figure 22

Figure 22. The autoradiographs resulting from Northern blots to test the size of messages to which the oligonucleotide probes hybridize. The probes being used are as described in the text in Section 3.2.2. (A) CNP, (B) MBP , (C) Tubulin, (D) Actin, (E) PLP/DM-20.
The markers are 2.0kb and 5.0kb.

After the third wash in 3xSSC, 0.1% SDS the filter had very little radioactivity remaining and was put under film.
No band was found on the autoradiograph and so the protocol above was repeated using a lower wash temperature of 37°C. This time a single band was detected on the autoradiograph and when repeated this technique of washing and hybridizing at 37°C proved successful for all tested oligonucleotide probes (see Section 3.2.2. and Fig. 22 for these results).

3.2.2. RESULTS OF NORTHERN BLOTS

All Northern blots were performed as described in Sections 3.2.1. and 2.4.1. These blots were performed to confirm that the oligonucleotide probes hybridized to only mRNA species of the size intended.
Figure 22 shows the size of messages detected by the following probes:

a) ATC TTG AAG GAA AGG GAA - anti-CNP

b) CGC ACC CCT GTC ACC GCT - anti-MBP

c) AAC ATT CAG GGC AGC GAT - anti-α-tubulin

d) TGG GGT GTT GAA GGT CTC - anti-β-actin

e) GAA ATA GGT CTC AAT TAG CTT TTC TGT ACC - anti-PLP/DM-20

The probes are described in more detail in Section 3.3.3., however their intended targets and a rough guide to their specificity are recorded below; according to the results generated in Fig. 22.
Probe A is specific for rat CNP and detects two messages; 2.4 and 2.8 kb as observed by Bernier *et al* (1988).
Probe B is specific for rat MBP exon I as described by Roach *et al* (1983). Since the probe is complementary to exon I it is able /...

is able to detect all species of MBP messages present (see Section 1.3.2 for details of MBP differential splicing). The only message detected is around the 2.2kb size - as observed by Newman et al (1987). It is particularly interesting to note that although there should be 4 messages present, only one band occurred on the autoradiograph. However the band detected was quite diffuse and since there is very little difference in size between the four messages this single band is quite probably four bands so close together that they seem to merge into one band.

Probe C is specific to rat α-tubulin and detects two messages of around 2.4 and 1.8 kb - as described by Villasante et al (1986).

Probe D is specific to rat β-actin messages and detects a single band of 2.0kb, which is in agreement with the observation of Cleveland et al (1980).

Probe E is able to detect both PLP and DM-20 messages from rats. Two message sizes are detected by this probe; 1.6 and 3.2 kb, as observed by Milner et al (1985). While this probe detects both PLP and DM-20, the autoradiograph pattern is the same as observed using either a PLP-specific or DM-20-specific probe (data not shown). Like the MBP probe it is likely that the PLP/DM-20 probe is detecting 4 messages;

a) a PLP message of 1.6kb

b) a DM-20 message of 1.6kb

c) a PLP message of 3.2kb

d) a DM-20 message of 3.2kb

Hence some of the messages are of such a similar size to each other that there are only two bands observed on the autoradiograph.

3.3. ASSOCIATION OF MESSAGES WITH THE CYTOSKELETON

3.3.1. CHOICE OF HYBRIDIZATION TECHNIQUE

Primarily one of two methods may be used to analyse messages in vitro; dot blots or Northern blots. If estimation of message size is an important factor in the work then it is vital to use Northern blots. However to simply check for the presence/absence of a message in a particular RNA fraction , dot blots are both more convenient and produce results more rapidly. The only proviso for use of dot blots is that the probe being used has already been proven to be specific for the message or messages being investigated.

Once a probe has been shown to be specific for a message, use of dot blot techniques is perfectly acceptable. In addition this method eliminates the need for running gels, the problems of transferring nucleic acids from gel to filter and in addition requires less filter space. Thus as many as six or seven dots may be placed on the filter surface area needed for a single gel lane.

The only problem for work with dot blots is ensuring the blot is confined to a small enough area, and that the RNA is not spread over too large an area of the filter. This is easily overcome by spotting on small aliquots of the sample and allowing them to dry before applying more sample.

3.3.2. DEVELOPMENT OF THE DOT BLOT TECHNIQUE

The basis of the methodology used here was developed by F. Ramalho-Ortigao (personal communications). The buffer used for both the hybridization and pre-hybridization consists of 7% SDS, 120µg/ml sonicated E. coli tRNA, 5xSSPE (20xSSPE= 3.6M NaCl /...

3.6M NaCl, 0.2M Sodium Phosphate, 20mM EDTA, pH 7.7). The most striking factor here is the simplicity of the buffer as compared to the buffer used for Northern blots. However the degree of background generated is very low, largely due to the very high SDS content, and using high wash temperatures eliminated the likelihood of non-specific hybridization.

The method used by Dr. Ramalho-Ortigao involved spotting 1.0μg of total RNA onto nitrocellulose. This was done in aliquots of at most 1.5μl and allowing the aliquots to air-dry before applying further samples. Nitrocellulose filters were baked at 80°C in a vacuum oven for 2 hours. Baked filters were pre-hybridized in the buffer described above for 2 hours at 37°C. Pre-hybridization was performed in seal-able freezer bags in a rocking water-bath, using 0.5ml of buffer per cm² of filter. At this point it is particularly important to ensure that air bubbles in the bag are kept to a minimum and in particular that any small air bubbles do not lie above the filter.

Hybridization was performed using the pre-hybridization buffer and required the addition of 10pmoles of probe per ml of buffer. A probe specificity of around 10^5cpm/pmole was used for a ^{32}P-kinase labeled oligonucleotide. Hybridization was carried out at 37°C for 3 hours in the same freezer bag as before, again ensuring a minimal bubble content.

Two washes of two minutes were performed at 37°C in 2xSSPE, 0.1% SDS followed by a two minute wash at (T_m-5)°C in 2xSSPE, 0.1% SDS. T_m is defined by the following equation:

T_m=4x(number of G+C)+2x(number of A+T)

However if the value of T_m exceeded 60°C, T_m was taken as 60°C for practical purposes.

Washed /...

Washed filters were allowed to air dry before being put under film for autoradiography.

The protocol descibed above was followed subject to two alterations.

1) When spotting on samples, drying was accelerated by use of a hot air stream from a hair-drier,
2) Instead of freezer bags, hybridization and pre-hybridization were performed in tightly sealed tubes. Tubes were placed in a sandwich box and allowed to roll on a rocking tray. This rolling motion ensured that the entire surface area of the filter was constantly being kept in close contact with buffer. Hence the problem of removing of bubbles was eliminated. This has a dual benefit as squeezing out bubbles can be difficult and also it prevents the possibility of the outside of the bag becoming contaminated with ^{32}P - and so increases safety.

Other points of note experimented with in this method were times of baking, pre-hybridization and hybridization. In all cases the times quoted are minimum times and may readily be exceeded.

Baking for less than 2 hours could on occasion lead to a reduced signal being detected. Additionally, care must be taken not to bake at temperatures in excess of 80°C in an oven which has not been evacuated as nitrocellulose readily combusts under such conditions.

As a negative control the sense oligonucleotide

ACC ACC ACA GCT GAG AGG G

which is the same as the RNA sequence found at nucleotides 601-619 of rat β-actin was used. This consistently provided no signal.

3.3.3. DISTRIBUTION OF MYELIN MESSAGES BETWEEN THE CYTOSKELETAL AND NON-CYTOSKELETAL COMPONENTS

As already discussed, the translation process takes place either on free polysomes or on membrane-bound polysomes (Section 1.4.4.). Free polysomes tend to be associated with the CSK fraction (Fey et al, 1986) and membrane-bound polysomes tend to be found in the non-cytoskeletal or soluble fraction (SOL), although there is association of some areas of the endoplasmic reticulum with the microtubules.

Six different oligonucleotide probes are described here:

i) CNP specific probe made to be complementary to nucleotides 40-57 of rat CNP messages;
ATC TTG AAG GAA AGG GAA

ii) MBP specific probe made to be complementary to nucleotides 163-180 of the rat MBP messages. This is part of exon I of the MBP gene and so is able to detect all MBP messages;
CGC ACC CCT GTC ACC GCT

iii) α-tubulin specific probe made to be complementary to nucleotides 832-849 of rat α-tubulin messages;
AAC ATT CAG GGC AGC GAT

iv) β-actin specific probe made to be complementary to nucleotides 370-387 of rat β-actin messages;
TGG GGT GTT GAA GGT CTC

v) PLP specific probe made to be complementary to nucleotides 361-390 of rat PLP messages. This sequence of the message is specific to PLP, being spliced out of the DM-20 messages.
TTG ATG TTG GCC TCT GTA ACC CCT CCC CTT

vi) a probe designed to be complementary to nucleotides 331-360 /...

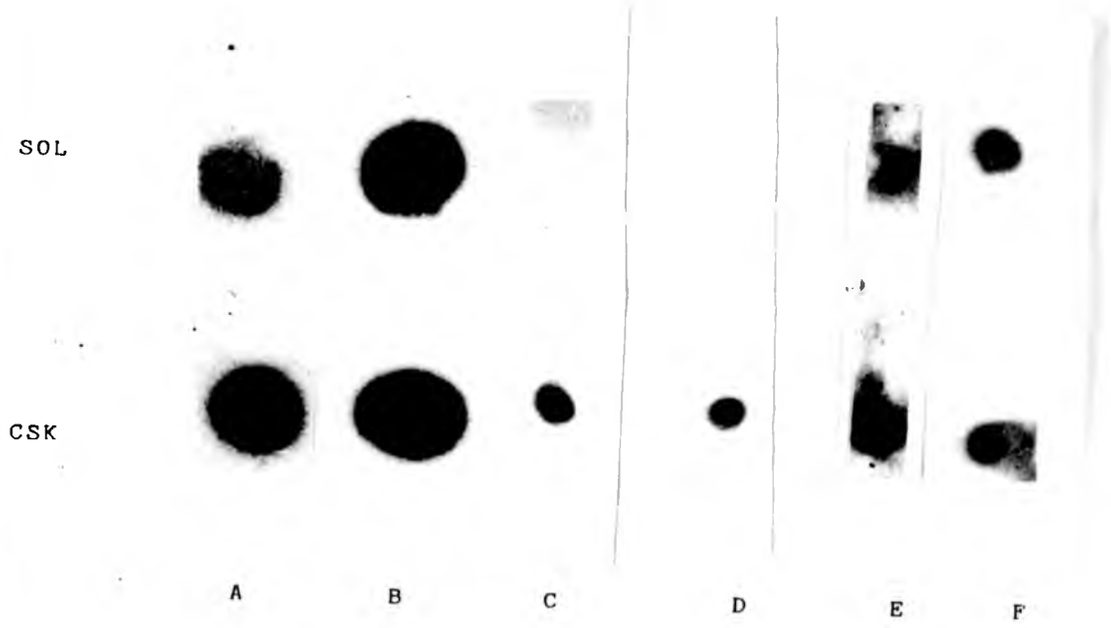


Figure 23

Figure 23. The distribution of various messages between the CSK and SOL RNA fractions. In each case the upper dot is the SOL fraction (where detected) and the lower dot is the CSK fraction.
Probes used are as follows; (A) CNP, (B) MBP, (C) Tubulin, (D) Actin, (E) PLP, (F) DM-20.

331-360 of the DM-20 message. However this sequence is also complementary to parts of the PLP message; nucleotides 331-345 and nucleotides 451-465. However it was hoped that the intervening 105 nucleotides would prevent any hybridization of the probe to PLP messages.

GGT GAT GCC CAC AAA CGT TGC GCT CAG GCC

Figure 23 demonstrates that the CNP message is found in both the CSK and the SOL fractions, with the considerably larger portion found in association with the cytoskeletal fraction. This association of the CNP messages with the cytoskeletal fraction is as expected for a message which is translated in association with free polysomes (Gillespie et al, 1990b). In addition the result supports the observation of Gillespie (1988), where it was noted that cytoskeletally associated RNA when translated produced considerably more CNP than the RNA from the non-cytoskeletally associated pool of RNA.

Like the messages encoding CNP, those encoding MBP are also found in the CSK and SOL fractions. However the distribution of the messages between the two fractions is much more evenly balanced. The fact that the MBP messages are more evenly distributed between the two fractions is in keeping with Gillespie (1988). Here it was found that the in vitro translational products of the MBP messages were found in fractions translated from both the cytoskeletally and non-cytoskeletally associated RNA pools.

Hence it appears that MBP messages capable of translation are found in association with the non-cytoskeletal and the cytoskeletal /...

cytoskeletal pool. However having the capacity to be translated *in vitro* says nothing about the *in vivo* status of messages, as they may have their capacity for translation masked in some way.

Since the dot blots are detecting all MBP messages nothing may be said about the distribution of the various messages between the CSK and SOL fractions. However the translations of Gillespie (1988) showed that the message encoding the 21.5kDa MBP may have a tendency to associate with the CSK. However this is only a reflection of those messages which retain the capacity to translate *in vitro*.

Since messages encoding the MBPs are translated on free polysomes (Colman *et al*, 1982), this suggests that the MBP messages which are associated with the SOL fraction are existing in one of two states:

i) messages translated on free polysomes which are not CSK-bound,

or ii) messages which are capable of translation, but are currently latent as regards translation.

Alternatively some combination of the two is possible. This depends on the efficiency of translation of non-CSK-bound free polysomes.

Both actin and tubulin encoding messages are found exclusively associated with the CSK. In both cases this seems logical as it permits rapid integration of the nascent proteins should they be required. The CSK exclusive nature for tubulin at least is in keeping with the observation of Black *et al*, (1986), where it was found that the tubulin messages of neurons are CSK exclusive.

Finally /...

Finally the messages encoding PLP and DM-20 which are translated on membrane-bound polysomes (Colman et al, 1982) are found in both the CSK and SOL pools. No obvious difference is detected as regards one message or the other having a stronger tendancy to associate with one particular pool or the other.

It should be noted here that although Colman et al (1982) documents that PLP translation occurs on membrane-bound polysomes, there is no actual record of the type of translation utilised by DM-20 messages. However since only 105 nucleotides of a difference has been reported between the two types of message , it has been extrapolated that the messages encoding DM-20 are translated in the same manner as those encoding PLP messages - i.e. on membrane-bound polysomes.

In addition this observation of PLP and DM-20 messages being associated with the CSK is in keeping with the observation of Terasaki et al (1986), where it was reported that the endoplasmic reticulum shows some degree of association with the cytoskeletal element of the cell - most probably this is the microtubules.

3.4. DEVELOPING A PROTOCOL FOR in situ HYBRIDIZATION

3.4.1. ENSURING SUCCESSFUL HYBRIDIZATION

It is now generally accepted that to investigate the sub-cellular distribution of messages within any particular cell type requires a fine-tuning of the technique to optimise the conditions for successful in situ hybridization.

Even having developed one particular technique for a cell type does not ensure its successful utilisation by other researchers, as experimenters technique, source of chemicals, batch lots chemicals and state of cells (sections or tissue cultures) can prove major factors in methodology.

As a starting point the logical place seemed to be the use of the first technique devised to investigate the spatial distribution of specific mRNAs within intact cells (Lawrence and Singer, 1985; Lawrence and Singer, 1986).

Cells were fixed for 15 minutes using 4% paraformaldehyde in PBS/5mM $MgCl_2$ and were stored in 70% ethanol at 4°C until required. Cells stored in 70% ethanol were rehydrated in PBS/5mM $MgCl_2$ for 10 minutes.

Cells were incubated in 50% formamide, 2xSSC (20xSSC is 3M NaCl, 0.3M tri-sodium citrate) at 60°C for 10 minutes prior to hybridization. Hybridization was performed at 37°C for 3 hours in 50% formamide, 2xSSC, 0.2% BSA, 10mM vanadyl sulphate ribonucleoside complex, 10% dextran sulphate, 1 mg/ml E. coli tRNA, 1 mg/ml salmon sperm DNA, 10mM DTT and 0.4 pmoles of ^{35}S labeled anti-tubulin probe (AAC ATT CAG GCC AGC GAT) - for description of this particular probe see Section 2.4.6.

Cells were then washed for 30 minutes under each of the following conditons:

i) 2xSSC, 50% formamide at 37°C

ii) 1xSSC, 50% formamide at 37°C

iii) 1xSSC at room temperature

Cells were then dehydrated through 70%, 95% and 100% ethanol, dried in air and the coverslips where cells were grown were mounted onto microscope slides (cell side up) using clear nail polish.

Slides were dipped in Ilford K5 emulsion using a Kodak No. 1 dark red safe light filter as sole light source. Slides were exposed in a light-tight container in the presence of Silica gel for 7 days. Slides were processed through D-19 developer for 2 minutes, and fixed in 30% sodium thiosulphate for 3 minutes followed by a 30 minute wash in ddH_2O. Material to be examined was mounted in 0.1xPBS/90% glycerol and viewed by microscopy.

This protocol follows the basic design of that described by Lawrence and Singer (1986), with a few differences :-

i) The probe being added is as a specific molarity rather than as a weight of partially digested cDNA per volume, however the two have similar amounts of radioactivity added.

ii) Ilford K5 emulsion was used instead of Kodak NTB-2. The two are found to have very similar detection levels and grain sizes.

iii) The development of the emulsion is different but is that recommended by Ilford for their product.

These three points are very important to note as they are the only differences in the technique and are relatively minor considerations. However these were maintained throughout the rest of the <u>in situ</u> hybridization work descibed here.

This technique yielded no labeling on the three occasions it was attempted, and so it seemed that this set of conditions must be modified in some way to achieve successful hybridization. The most /...

The most obvious criterion to vary is the time of exposure in the light-tight box. Even after 2 weeks no silver grains could be detected which could in any way be construed as being a positive signal.

After checking with the technical services provided by Ilford, the hybridization was repeated and this time the light-tight box was sealed with masking tape to minimise the likelihood of any moisture coming in contact with the emulsion inside. Again only a few silver grains could be detected randomly distributed around the cover-slip. However despite not having generated a successful hybridization technique at least the problem seems to have been narrowed down to the actual hybridization technique rather than the emulsion and development.

This was confirmed by spotting some ^{35}S labeled probe onto a slide, allowing it to dry in air and then spotting on some emulsion and allowing it to dry before exposing for 24 hours and developing. This time a large mass of silver grains was detected. Hence it was clear that the emulsion technique is satisfactory.

From this spotting technique one other modification arose; dipped slides were allowed to air dry under the No 1 filter for 2 hours before being placed in the light-tight box and sealed up.

A number of other *in situ* hybridization techniques were performed as described in the literature. Those methods used included; Eng *et al* (1986), Bloch *et al* (1986), and Hafen *et al* (1983).

In all cases no specific signal was detected, and only a few isolated silver grains were observed on any coverslip. All techniques /...

techniques were repeated at least three times before being dismissed in their current form.

However one fact did become clear from use of these techniques; dehydration in ethanol must be performed gradually. Cells exposed to 70% ethanol often took on a distorted form and so any dehydration was performed via 50% ethanol, 70% ethanol, 80% ethanol, 90% ethanol, 95% ethanol, 99% ethanol and finally 100% ethanol.

Cells being rehydrated were rehydrated through the above process in reverse. These cells when viewed by phase contrast microscopy appear unchanged. While unchanged appearance of cells does not give any assurance that there has been no change, it is unlikely that distorted cells can be viewed as being acceptable for further studies.

The first positive results generated were at the University of Ulm using the anti-tubulin probe described in Section 3.3.3. (AAC ATT CAG GGC AGC GAT) on brain sections using the protocol described in Jirikowski et al (1988) and Ramalho-Ortigao et al (1988). Silver grains were found in clusters suggesting that the messages are concentrated in a particular area - most probably the centre of the cell.

The protocol used was for sections floating in reaction vials and is described below.

Prehybridization was performed in 50% formamide, 5xSSPE, 0.9% NaCl, 2x Denhardt's Solution (see Section 2.4.1.), 1 mg/ml BSA, 100μg/ml sonicated E. coli tRNA for 2 hours at 37°C. After prehybridization ^{32}P labeled probe was added straight to the prehybridization mix (1 pmole per ml). Hybridization was carried out overnight at 37°C and was followed by a 15 minute wash in 5xSSPE (1xSSPE = 180mM NaCl, 10 mM Sodium phosphate /...

10 mM Sodium phosphate pH 7.0, 1mM EDTA), and a 10 minute wash in 0.9% NaCl, PBS, 0.05% Sodium azide.

Sections were floated onto microscope slides and dehydrated before dipping in emulsion

It should be noted that the probe here was a 5' labeled oligonucleotide and that the radioisotope was ^{32}P. This is in contrast to the previous attempts were probes which were 3' labeled and the radioisotope was ^{35}S.

^{32}P has the advantage of requiring a shorter exposure time, which allows experiments to be performed over a shorter period. However it is essential to keep the emulsion much thinner in ^{32}P experiments due to the longer track length of the emitted electron on decay of the isotope.

Initial attempts to reproduce the technique at Stirling using cells from tissue culture as opposed to sections proved unsuccessful. However at least the anti-tubulin probe had been shown to cause successful *in situ* hybridization.

At this point the decision was made to increase the effective concentration of the probe in the hybridization solution by including 10% dextran sulphate (Lawrence and Singer, 1985). Lawrence and Singer suggested that inclusion of 10% dextran sulphate can cause as much as a 3 fold increase in signal and possibly also introduces some degree of protection to messages. Since this technique was being developed for cells grown in tissue culture which are not part of a confluent monolayer there is an obvious difference between the tissue culture cells and those in brain sections. Hence reducing the effective volume by increasing the effective concentration is obviously beneficial.

This produced /...

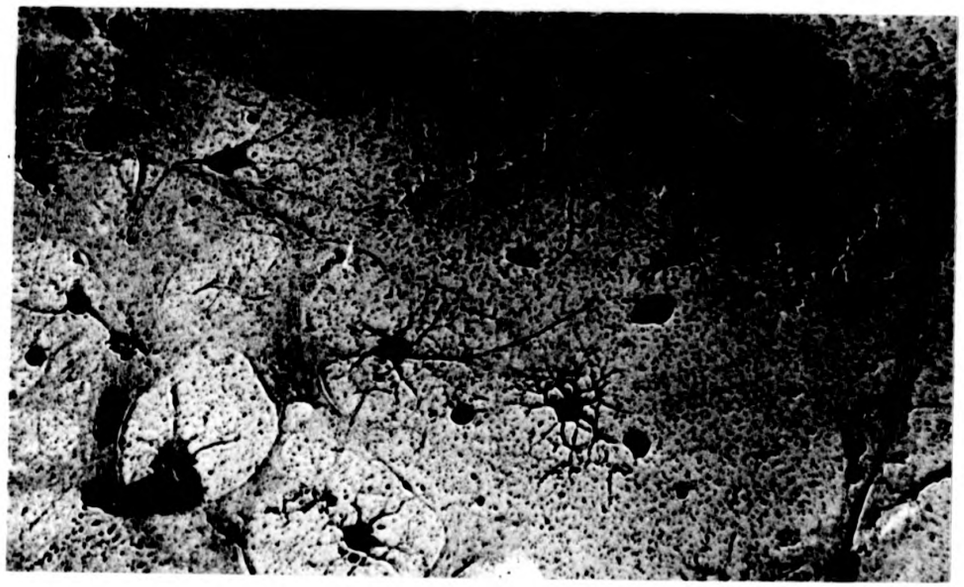


Figure 24

Figure 24. Oligodendrocytes which have been probed using an anti-tubulin oligonucleotide probe. The centre of the cells is the area of most intense hybridization. Also present are some non-oligodendroglial cells which show a much reduced hybridization signal. These are most probably astrocytes.
The size bar = 100μm.

This produced some degree of success, but the level of background was possibly slightly higher than had been hoped for (Fig. 24). To reduce the background a series of washes were performed using only the NaCl, PBS, azide wash buffer for a longer period than that previously used. The level of ^{32}P coming off in any wash was monitored using a Geiger counter and washes of around 5 minutes were found both convenient and effective. 5 minute washes allowed a number of slides to be washed on a rota basis and times longer than this tended to only minimally increase the amount of ^{32}P being washed away at any one time. After ten such room temperature washes little, if any, ^{32}P was being washed away. A 5 minute wash was performed at 37°C to remove any partially hybridized probe. This was followed by one final wash at room temperature to remove any dissociated probe from the previous wash which was now lying loose.

Cells were then dehydrated using the ethanol stages described previously and coverslips were fixed cell-side up on slides using nail polish. These slides are now ready to dip in emulsion. This gave a much better signal:noise ratio (Fig. 25).

Negative controls were used consisting of a 1 hour incubation in 100μg/ml RNase at 37°C prior to prehybridization for all in situ hybridizations.

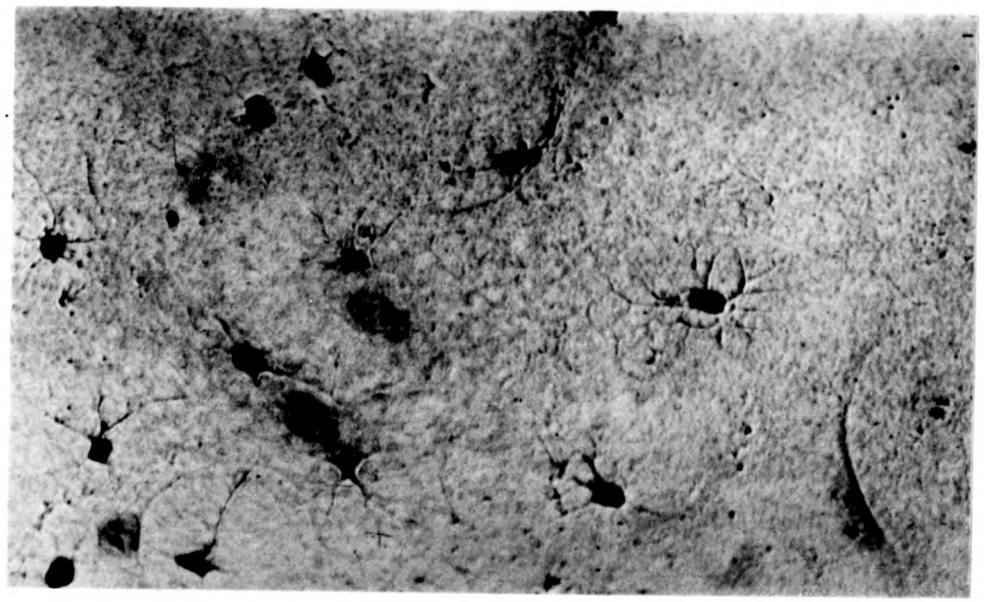


Figure 25

Figure 25. Oligodendrocytes which have been probed using an anti-tubulin oligonucleotide probe. The centre of the cells is the area of most intense hybridization. It is clear that the level of background darkness is reduced relative to that observed in Figure 24.
The size bar =100μm.

3.4.2. DEVELOPMENT OF JOINT HYBRIDIZATION AND IMMUNOCYTOCHEMICAL TECHNIQUES

Initial results from the in situ hybridization work from the anti-tubulin and anti-PLP probes detected signals in centralised clumps, which were assumed to be cell bodies. However the fact that phase contrast microscopy and Nomarski optics microscopy proved difficult to achieve in conjunction with the emulsion dipping technique generally resulted in a problem providing that the rest of the cell had actually been preserved (Fig. 26). The best way to prove that the cell has had its structure maintained is to prove that the morphology is retained by use of antibodies and immunocytochemical methods. By this technique not only will much more of the cell's shape be revealed according to the protein distribution found by Wilson and Brophy (1989), but also using antibodies specific for myelin proteins it is possible to prove that the cells being probed are oligodendrocytes.

As already discussed in Section 2.2.3., the early cell fixation procedure required modification before it could be applied to immunocytochemical work.

The next problem to deal with was that of which technique to employ first; hybridization or immunocytochemistry. To tackle this problem oligodendrocytes were probed using Rhodamine-Phalloidin to check that the conditions used for in situ hybridization gives rise to an immunocytochemical signal which is representitive of the signal obtained in the absence of the hybridization protocol.

In addition the in situ hybridization technique was performed using 0.2% gelatin as a blocking agent instead of 5x Denhardt's solution. A comparison of in situ hybridization results was generated /...

(A)

(B)

(C)

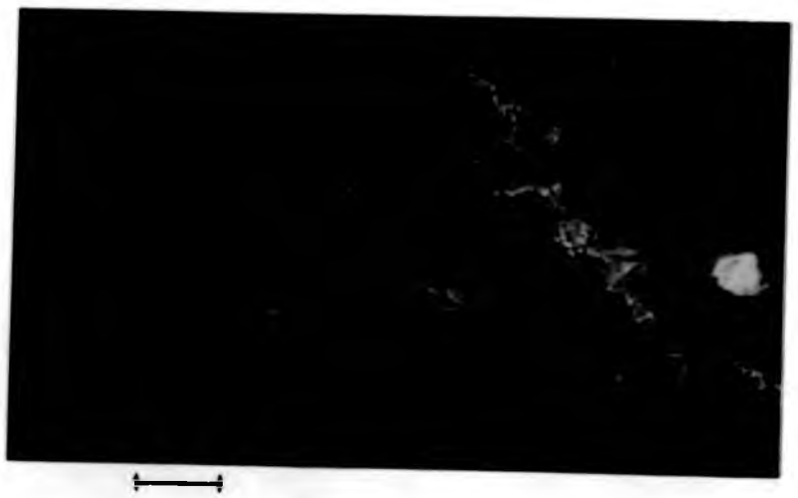


Figure 26

Figure 26. Use of Nomarski optics proved problematic when used in conjunction with cells which had been dipped in Ilford K5 emulsion.

(A) This shows a cell which resembles an oligodendrocyte, based on its morphology, and at least three other cells around it. These cells have been viewed by use of Nomarski optics and have not been dipped in emulsion. A number of processes can readily be seen extending from the cell body. In addition many of the finer, more peripheral processes may be seen.

(B) An oligodendrocyte probed using <u>in situ</u> hybridization for its PLP messages. Clearly the silver grains lie in the centre of the cell. In addition a certain amount of brown staining is detected in the processes of the oligodendrocyte and in the surrounding non-oligodendroglial cells. This brown staining is not to be confused with the black observed for silver grains in the emulsion. These cells were viewed by bright field microscopy.

(C) This shows the same field as (B) but the cells have been viewed by Normaski optics rather than bright field. Clearly the same degree of detail achieved in (A) is not possible. Indeed there appears to be no benefit over use of bright field. This was also observed when phase microscopy was used.

Size bar = 20μm

Figure 27

Figure 27. This figure shows cells which have been probed for their tubulin message distribution by use of <u>in situ</u> hybridization. This figure can be compared with Fig. 25, and quite clearly the two figures demonstrate a similar distribution of messages; messages being primarily found in the cell body.

However one important distinction must be made between the two figures:- in Fig. 25 the blocking agent used was Denhardt's solution; in Fig. 27 the blocking agent used was 0.2% gelatin.

As already intimated, there is no clear difference in message distribution as detected uding the two different blocking buffers. Therefore it was concluded that either buffer was acceptable for <u>in situ</u> hybridization studies, but in order that joint hybridization and immunocytochemical work might be perfomed it was decided to favour use of 0.2% gelatin as a blocking agent.

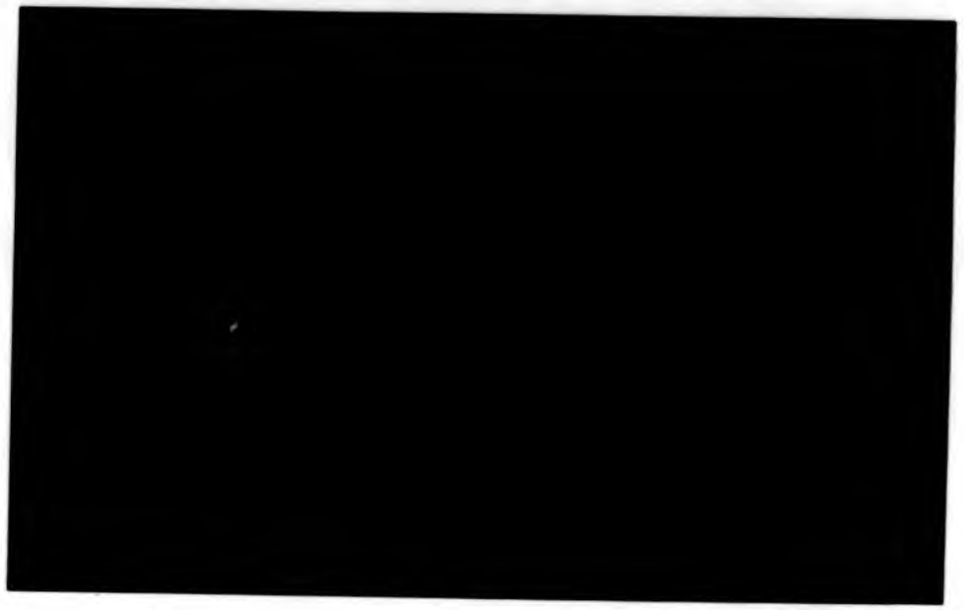
Size bar = 100μm.

generated using the two blocking components in the pre-hybridization buffer. From Figs. 25 & 27 there is no obvious difference in the distribution pattern of the messages as determined by the two methods, suggesting that substituting 0.2% gelatin for 5x Denhardt's solution is an acceptable change.

The change to 0.2% gelatin is based upon the fact that the 2% gelatin solution which is used as a stock solution has been autoclaved to minimise the effects of ribonucleases. There is still the possibility that the antibody solution used in the immunocytochemistry will contain ribonucleases as this solution cannot be autoclaved for fear of denaturing the antibodies. However when this work was performed using the antibodies this did not appear to happen.

To check the effects of the hybridization solution on the signal obtained from immunocytochemical work the prehybridization treatment was performed either before or after incubation with Rhodamine-Phalloidin. The incubation with phalloidin was performed under the same conditions as used for normal antibody work and so could be assumed as giving representative results for any denaturation of signals. An advantage exists in using Rhodamine-Phalloidin; since it is already labeled there is no need for a second antibody to detect a primary antibody as for the antibodies raised against the myelin specific proteins. Obviously having to use a second incubation during development methods is to be avoided as much as possible as it provides another potential point for problems to occur. The ideal situation for methodological development is to keep the number of stages in the protocol to an absolute minimum, making it simpler to isolate the source of any problems which may result.

(A)



(B)

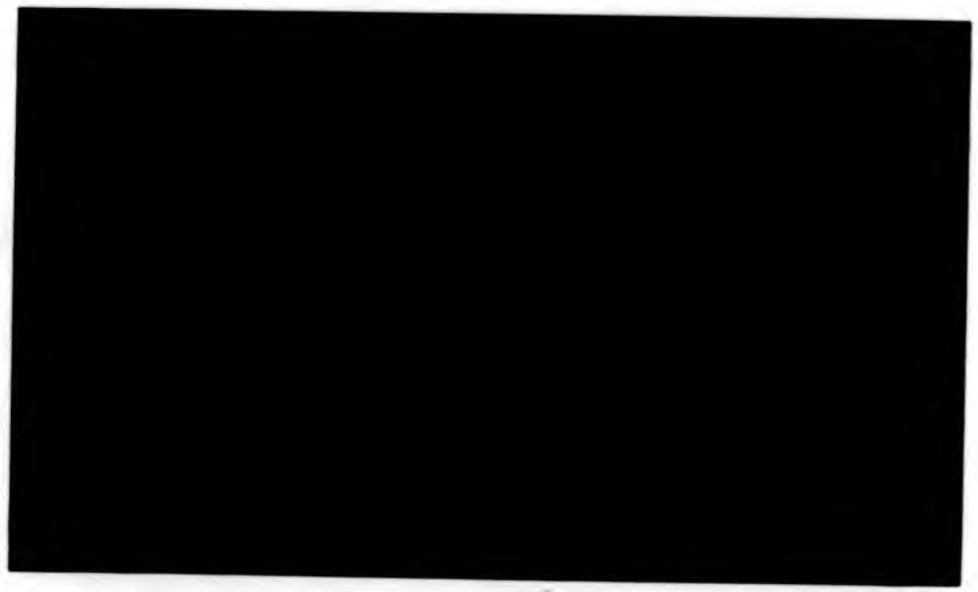
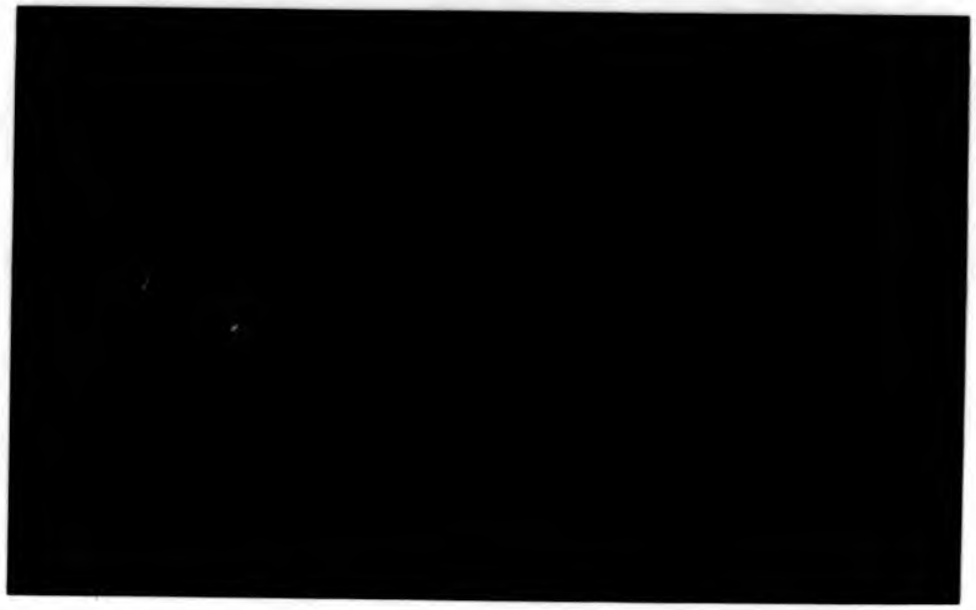


Figure 28

Figure 28. A comparison of the orders preferred for *in situ* hybridization and immunocytochemistry. (A) The *in situ* hybridization was performed before the immunocytochemistry. (B) The *in situ* hybridization was performed after the immunocytochemistry. In both cases only detection of filamentous actin, by use of Rhodamine phalloidin, is shown. It appears that using the hybridization technique first is detrimental in terms of immunocytochemical signal intensity, as the signal detected in (B) is both faint and also irregular relative to that described in Wilson and Brophy (1989) and also relative to (A) and also Fig.44A. Size bar = 20μm.

(A)



(B)

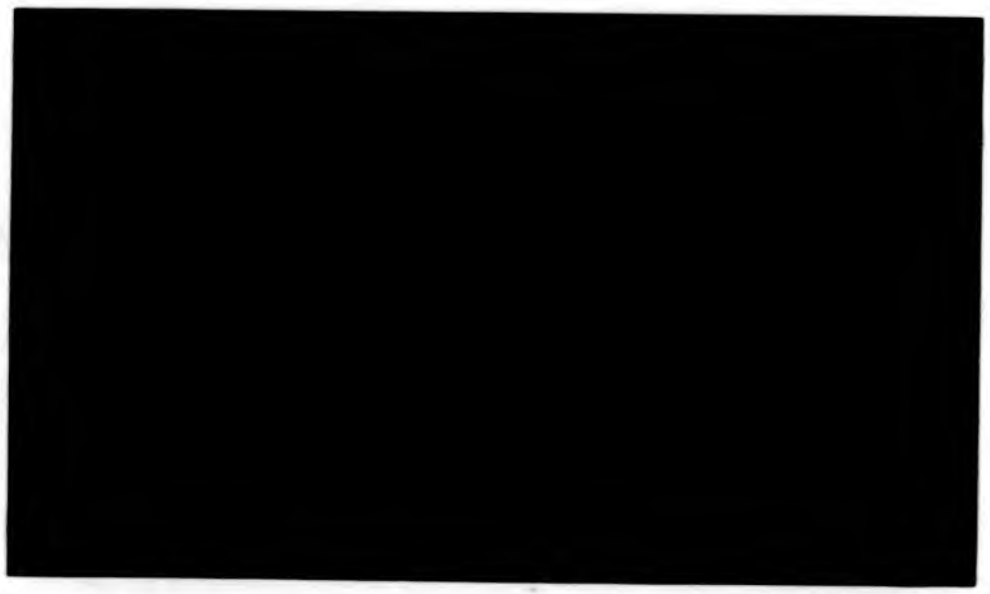


Figure 28

Figure 28. A comparison of the orders preferred for in situ hybridization and immunocytochemistry. (A) The in situ hybridization was performed before the immunocytochemistry. (B) The in situ hybridization was performed after the immunocytochemistry. In both cases only detection of filamentous actin, by use of Rhodamine phalloidin, is shown. It appears that using the hybridization technique first is detrimental in terms of immunocytochemical signal intensity, as the signal detected in (B) is both faint and also irregular relative to that described in Wilson and Brophy (1989) and also relative to (A) and also Fig.44A. Size bar = 20μm.

Figure 28 demonstrates that the use of the hybridization technique before the use of immunocytochemistry results in a poorer signal from the Phalloidin, presumably because the actin filaments are not quite in the same form as they were before hybridization.

However pre-hybridization after the immunocytochemistry led to a reduced signal, albeit fairly typical in distribution of that found in the absence of pre-hybridization treatment. Hence this order was repeated, but before pre-hybridizing, the cells were once again fixed as described in Section 2.2.3. This time a stronger signal was obtained. It is desirable to obtain as strong a fluorescent signal as possible as this facilitates detection of very faint signals. In addition exposure to the light source from the fluorescent microscope leads to bleaching and so fading of the signal. Hence the stronger signal reduces the time required for exposure to obtain a satisfactory photograph.

One final modification to the immunocytochemical methodology used routinely in the lab was the omission of phenylenediamine from the mounting media before performing microscopy. Phenylenediamine is normally used to reduce the level of fading of the fluorescent marker. However its inclusion in this work seemed to result in streaking of the emulsion (Fig. 29) which contained the silver grains. Hence a true picture of the distribution of the mRNA being investigated could not be obtained. Omitting the anti-fading agent did not seem to present a major problem as even several months later the fluorescence was still strong and easily detected. However as an additional precaution for faint signals, the absence of the anti-fading /...

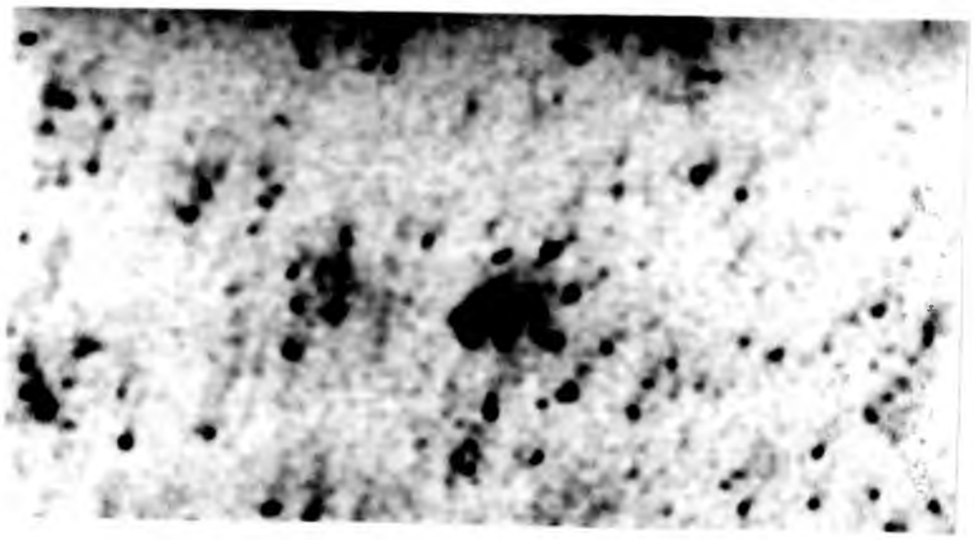


Figure 29

Figure 29. Including phenylenediamine in the mounting media is a standard procedure to reduce the fading effects of fluorescent compounds. However inclusion of this agent in the presence of the emulsion used for *in situ* hybridization leads to a stripping of the emulsion which appears as a streaking effect. In the case of this slide the streaking effect is so extreme as to mask the shape of the cell entirely. Size bar = 20μm

the anti-fading agent from the mounting media makes it all the more important that the signal intensity is maximised in the first instance by use of the second fixation step after the final wash step in the immunocytochemical part of the joint detection protocol.

The finalised methodology for joint immunocytochemistry and in situ hybridization is described in detail in the materials and methods chapter (Section 2.4.4.).

3.4.3. GUIDE TO in situ HYBRIDIZATION SPECIFICITY

Ideally the best way to check the specificity of any probe for in situ hybridization is to synthesize the same probe with a single mis-match in the sequence. However to do this for each probe is a very costly process. Instead it was observed that the two anti-actin oligonucleotides also showed a great deal of homology to the sequence required for a human β-actin probe.

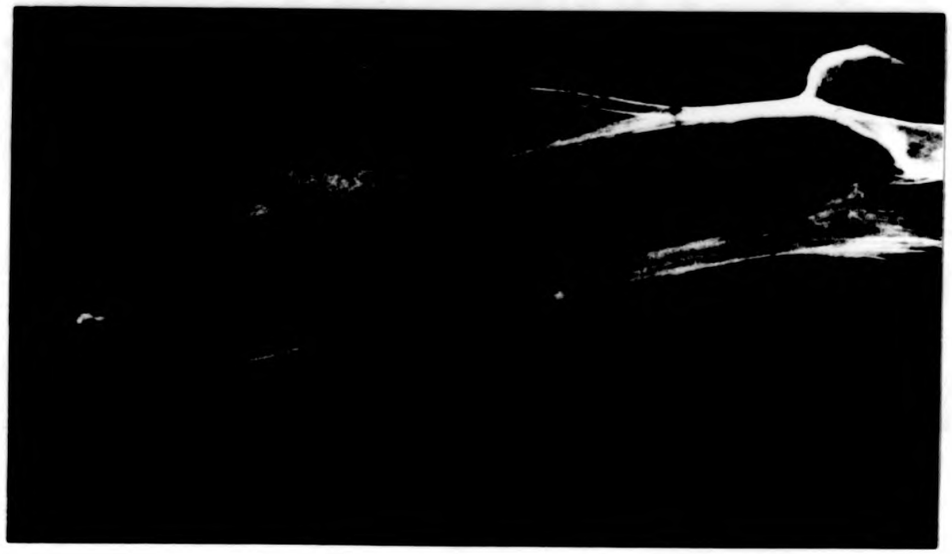
The sequence TGG GGT GTT GAA GGT CTC is 100% complementary to nucleotides 373-390 of both human and rat β-actin messages. However the sequence ATA GCA CAG CTT CTC TT is complementary to nucleotides 637-653 of the rat β-actin message, but shows a single mis-match at nucleotide 651 in the human β-actin message.

In theory both nucleotides should be effective probes for use with rat cells, but if the specificity of the probes is as high as hoped only the one with 100% complementarity will hybridise in human cells. The results of the probes being used in rat cells is dealt with in Section 3.5.3., but in summary both probes successfully hybridise to β-actin messages in rat oligodendrocytes.

Here the results obtained from use with the human fibroblasts available in the lab are presented. Figure 30 shows the presence of fibroblasts by detection with Rhodamine-Phalloidin which will detect filamentous actin. Labeling with Phalloidin was performed as described for immunocytochemistry in Section 2.4.4. and Phalloidin was diluted to a final concentration of 1% of the original concentration supplied by Sigma chemicals.

The first attempt at in situ hybridization using the protocol for oligodendrocytes /...

(A)



(B)

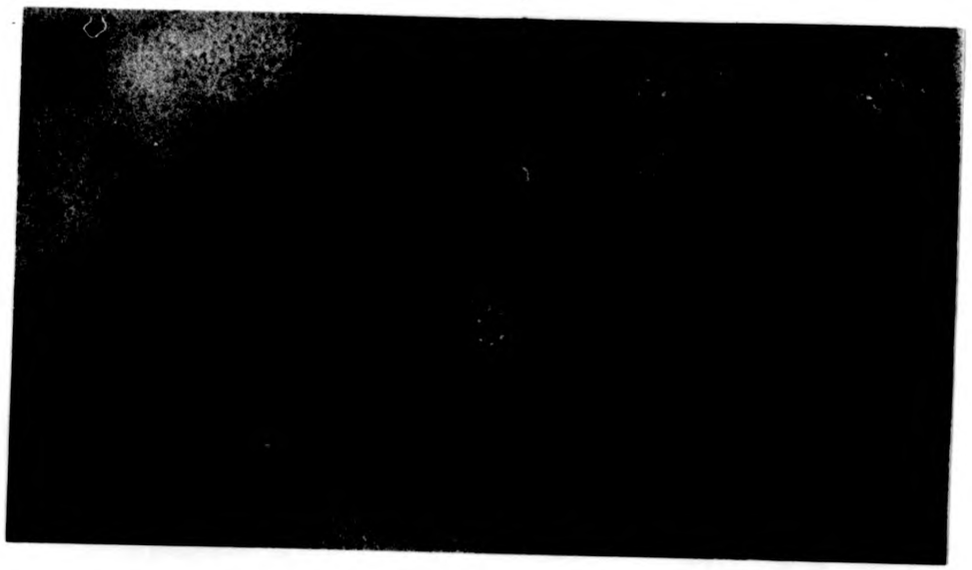


Figure 30

(C)

(D)

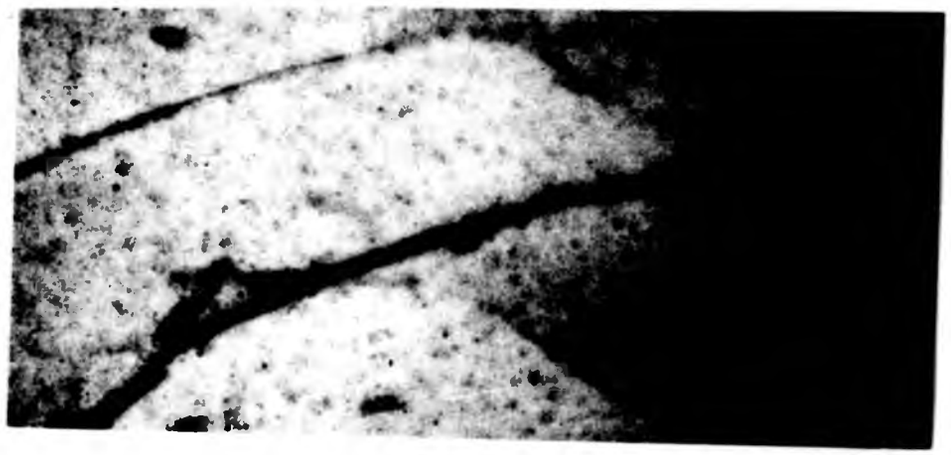


Figure 30

Figure 30. A comparison of the distribution of the degree of sensitivity of a single mis-match probe and a 100% complementary probe in human fibroblasts.
(A) The distribution of filametous actin within human fibroblasts as detected by Rhodamine-phalloidin and fluorescent microscopy.
(B) The same cells as in (A) but after probing with a probe which has a single mis-match to the human actin gene, as detected by autoradiography and bright field microscopy.
(C) The distribution of filamentous actin within human fibroblasts as detected by Rhodamine-phalloidin and fluorescent microscopy.
(D) The same cells as in (C) but after probing with a probe which is 100% complementary to the human actin gene, as detected by autoradiography and bright field microscopy.
Size bar = 10μm.

for oligodendrocytes proved successful. Since the fibroblasts were being used merely as a rough guide to the sensitivity of the method no optimisation studies were performed to try and improve upon the first successful hybridization.

It is quite clear that the 100% complementary sequence as a probe proved very successful, showing actin messages to lie in the peripheral areas of the cell - in agreement with the results of Lawrence and Singer (1986). However the probe which contains a single mis-match is unable to detect the actin messages in the cell.

The results presented here in conjunction with those in Section 3.5.4. suggest that there is a high degree of specificity contained in the technique.

It would of course have been better to use a single mis-match probe on the same cell type, preferably raised against the same sequence of nucleotides within the message. This however has the problem of using a probe which has not been successfully shown to hybridize to a message, which may reflect a problem in the synthesis of the oligonucleotide rather than an inability of the probe to hybridize at such high specificity. However this could be tackled to some extent by use of lower stringency Northern blots, although Northern blotting with oligonucleotide probes has proved difficult to achieve - even when using 100% complementary probes.

In addition a better situation would be a contrast of messages contained in the same cell type from different species. However since ultimately the work in this thesis is concerned with oligodendrocytes, the rat cells used were oligodendrocytes. However the use of human cells posed additional constraints

hence /...

hence human fibroblasts were used rather than oligodendrocytes. The fact that a sequence of the rat β-actin message is available for hybridization in the rat oligodendrocyte does not imply that it is going to be available for hybridization in the human fibroblast. However it does seem reasonable to assume that the message will be available for hybridization in the absence of any detailed knowledge of the message's interactions with proteins.

In summary the work presented in this section does not deal with a perfect control, but nevertheless it does suggest that the methodology being used in the in situ hybridization technique has a reasonably high degree of specificity.

3.5. SUB-CELLULAR DISTRIBUTION OF MYELIN PROTEINS AND THEIR MESSAGES

As already discussed in Section 1.4.6. a number of different myelin messages encoding myelin messages have already had their distribution investigated. However much of this work has been performed on brain sections rather than oligodendrocytes grown in tissue culture. As already discussed the morphology of an oligodendrocyte is not particularly conducive to work performed on sections as cutting through an oligodendrocyte transversely is liable to lose much of the cell.
This problem may be overcome by cutting and checking successive brain sections for a particular feature, but being able to place samples one on top of the other is no simple matter.
Hence a study of tissue culture grown cells was undertaken here.
In this work presented here the size bar is 10μm unless otherwise stated.
In addition to the black silver grains left in the emulsion after developing, on occasion there are also areas of light brown staining found over cells. This can prove problematic if photography is undertaken using black and white film, as often the whole cell appears as a dark area. However when colour film is used the brown colour can actually prove beneficial as the cell's shape in entirity is now observed, rather than only the areas of silver staining. In addition it is still quite clear where the signal is found relative to the brown background.
It is important to understand that this brown staining is quite different from the black silver grains and is not to be confused with a positive *in situ* hybridization signal.

3.5.1. NEGATIVE CONTROLS FOR in situ HYBRIDIZATION

Before considering the results of the in situ hybridization in any great depth it is important to clarify that any negative controls designed, actually produce the required negative results.

As already discussed in Section 3.4.3. the use of a single base mis-match probe against human fibroblasts resulted in no signal being detected. However using this probe on a rat oligodendrocyte to which it is a perfect probe resulted in hybridization (see Section 3.5.4.). This gives some degree of perspective to the sensitivity of the probes.

In a similar fashion, treatment with both RNase and also performing hybridization with a sense probe (i.e. one which contains the same sequence as the message itself, rather than being complementary to the message) also leads to no signal being detected.

However these results are the same as would be generated by use of hybridization techniques on RNA which has been extracted from the cell. A much more interesting form of negative control is to use the probe on cells which are not oligodendrocytes, but are found in the same cultures as being used.

Figure 31 shows a non-oligodendroglial cell which has been probed using the DM-20 specific probe. Clearly the cell is not the typical shape of an oligodendrocyte, and is probably either an astrocyte or a macrophage. In addition there is no evidence of silver grains lying over the cell, suggesting the DM-20 probe has failed to hybridize to any messages in this cell type. However as Section 3.5.6. demonstrates, this probe does have the capacity to hybridize to messages /...

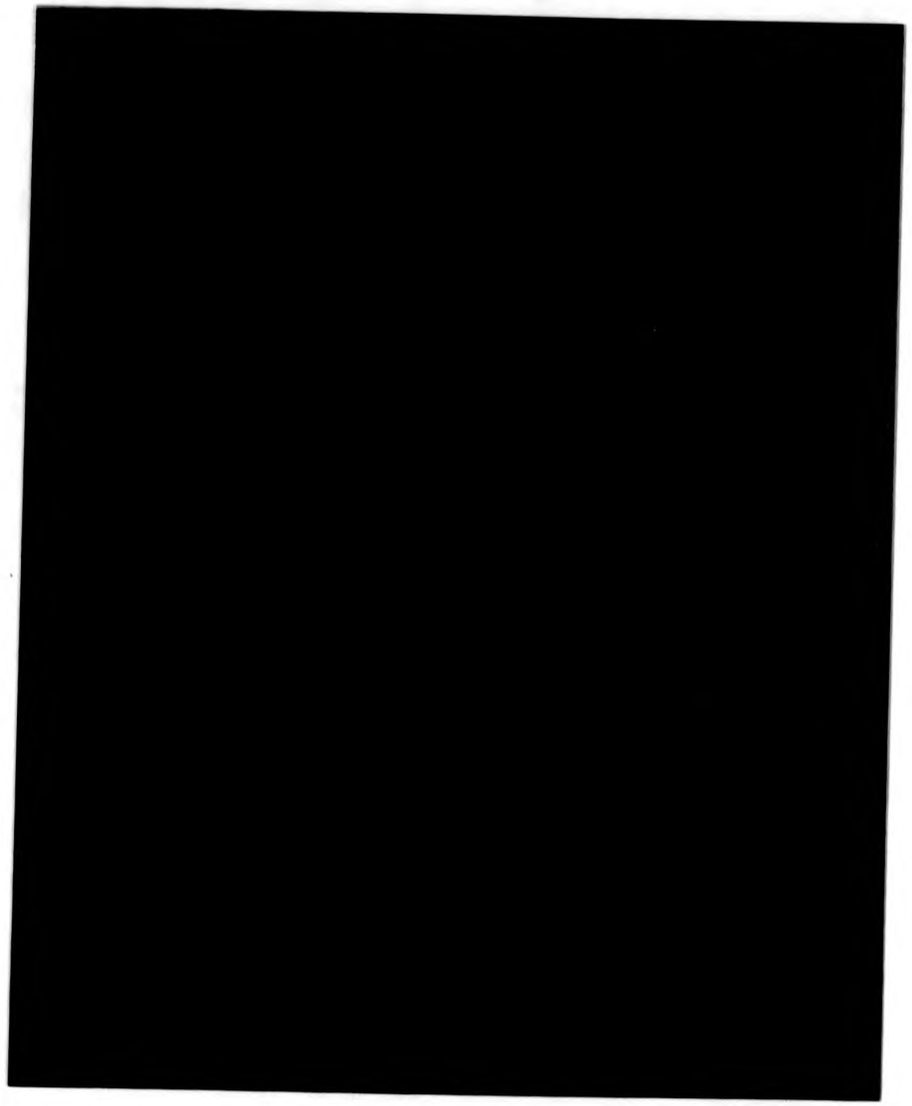
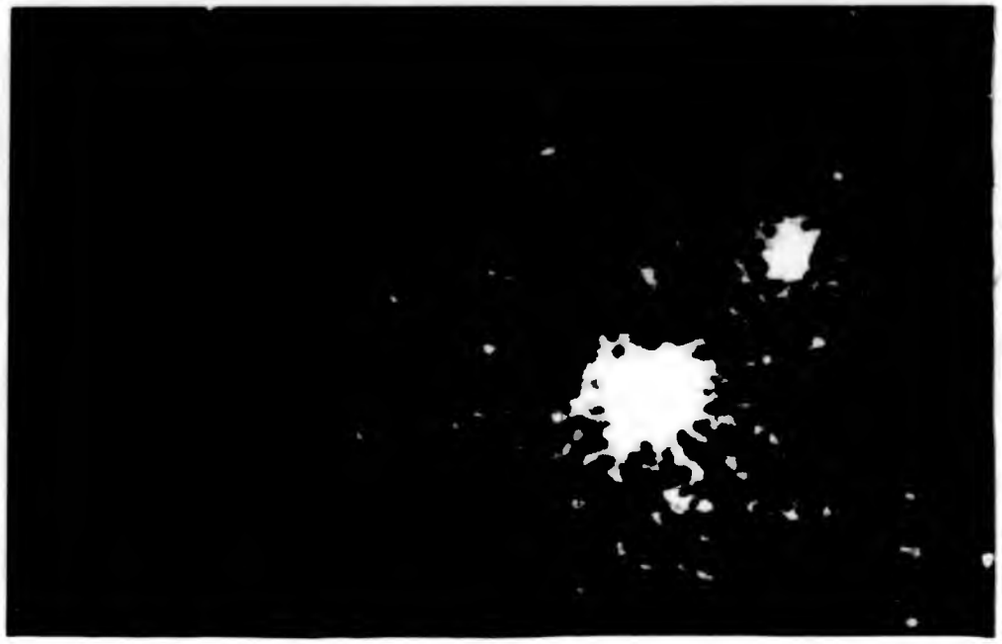
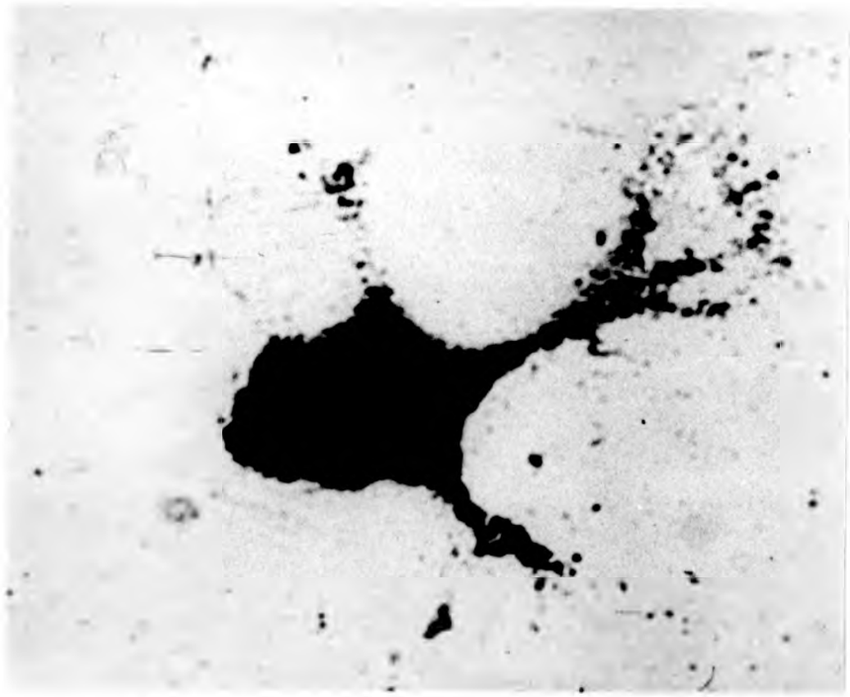


Figure 31

Figure 31. This figure shows a non-oligodendroglial cell which has been probed using the DM-20 specific probe. The brown staining outlines the cell's shape, but clearly no silver grains are present. Hence low levels of false positive signals can be expected using this probe.
Size bar = 10μm



(A)



(B)

Figure 32

Figure 32. A comparison of the use of two different anti-MBP probes being used to detect the sub-cellular distribution of MBP messages within oligodendrocytes.
(A) An oligodendrocyte probed by use of the oligonucleotide probe : CGC ACC CCT GTC ACC GCT. The distribution is in keeping with that observed in Figs. 11A & 11B; the messages lying mainly in the cell body and in the processes closest to the cell body.
(B) An oligodendrocyte probed by use of the anti-MBP probe : G ATC TTG GGT CCT CTG. Again the distribution of the messages is as described in the previous Figures.

Size bar = 10μm

3.5.2. SUB-CELLULAR DISTRIBUTION OF MBP MESSAGES

As already demonstrated in Figs. 11A & 11B the sub-cellular localization of MBP messages is primarily in the cell body and the major processes. This seems to be the case irrespective of the fixative used, or the blocking buffer used. However in all work done to investigate these parameters the probe used was the same :-

CGC ACC CCT GTC ACC GCT

Hence it is possible that the observation is in fact merely an artifact of one particular probe.

To further substantiate this observation cells were probed using a second anti-MBP oligonucleotide probe:-

G ATC TTG GGT CCT CTG

The typical cell distibution of messages as detected by use of these two probes is shown in Fig. 32, and again both show a similar sub-cellular distribution of MBP messages.

Hence it would appear that MBP messages tend to be found in the cell body and in the major processes - those which are in close proximity to the cell body, rather than the more distal minor processes. These observations are in keeping with the observations of Trapp *et al* (1987); Jordan *et al* (1989); Shiota *et al* (1989).

As yet another check on the authenticity of the observations detailed above probing was also carried out using riboprobes from a cDNA made available by Prof. I. R. Griffiths. The typical distribution pattern generated by this probe is shown in Fig. 33, and is in keeping with that already observed in Fig. 32 & 11. The only difference worthy of comment is that the use of a ^{35}S labeled probe seems to generate /...

Figure 33

Figure 33. An oligodendrocyte which has been probed using a ^{35}S labeled riboprobe which was transcribed from an MBP cDNA. The use of a ^{35}S labeled probe possibly results in slightly finer silver grains, but the general distribution pattern observed is the same as reported for cells which had been probed by use of an oligonucleotide probe; most messages lying in the cell body or in the major processes which lie around the cell body.

Size bar = 10μm

generate slightly sharper and more fine silver grains. However by keeping the emulsion layer sufficiently thin no real loss of resolution was found to result from use of a ^{32}P labeled probe.

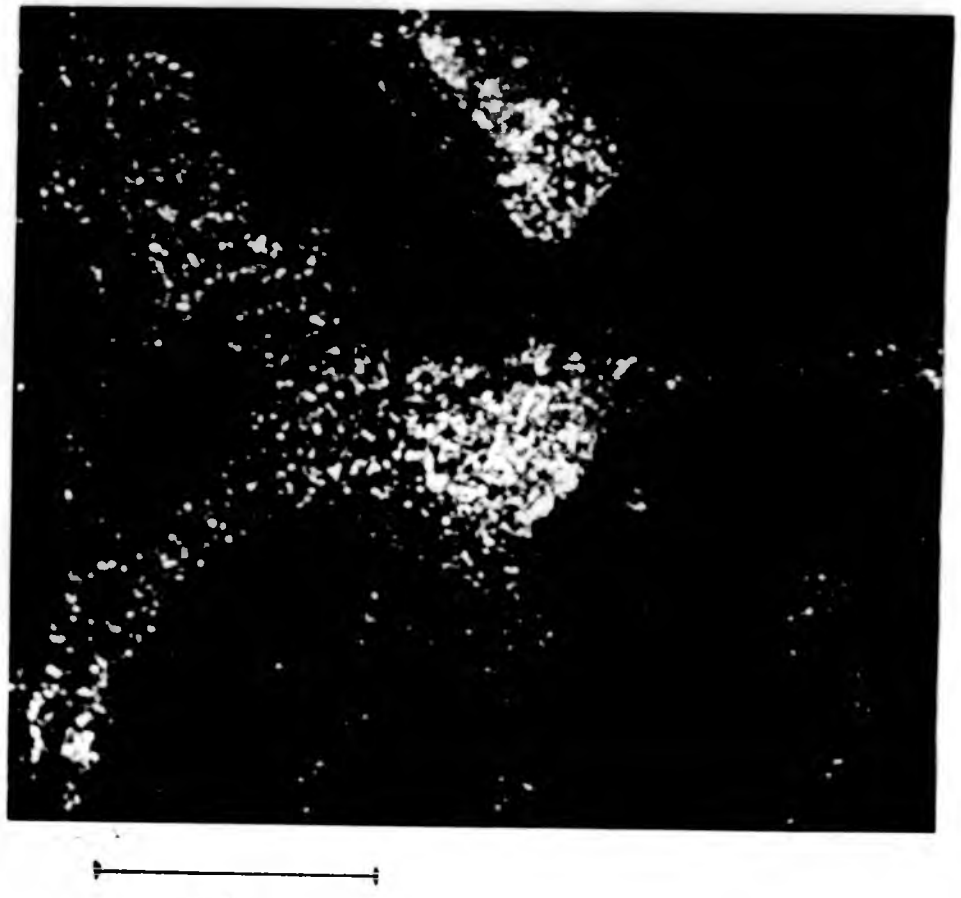
In addition to performing hybridization experiments on whole oligodendrocytes, the process of *in situ* hybridization was also performed on the cytoskeletal residue of oligodendrocytes. As already documented in Gillespie (1988) there appears to be a difference in the MBP message distribution between the CSK and SOL fractions on the basis of *in vitro* translation products. In Fig. 34 there appears to be a tendancy for the MBP messages associated with the CSK to lie slightly further from the centre of the cell, and no heavy silver staining is observed in the very centre of the cell body. This figure suggests that there is a difference in the sub-cellular localization between total MBP messages and those associated with the CSK.

Unfortunately in the current work such differences cannot be extrapolated to the level of differences in localization of differential splicing products, although a method to tackle this particular problem is discussed at length in Section 4, based upon a series of different oligonucleotide probes used in conjunction with each other to specify different splicing products from the MBP gene.

Figure 34

Figure 34. The cytoskeletal residue of an oligodendrocyte probed by use of the oligonucleotide probe : CGC ACC CCT GTC ACC GCT. There is a difference in the distribution of the MBP messages in the cytoskeletal residue relative to the intact oligodendrocyte :- the centre of the CSK residue no longer shows silver grains, instead the messages seem to be located in the more peripheral areas of the cell body and also in the major processes. Hence the distribution is the same as reported for the intact cell with the exception of the very centre of the cell body. This suggests that there is a difference in the sub-cellular localization of MBP messages relative to their distribution between the CSK and the SOL fractions.

Size bar = 10μm.



(A)

(B)

Figure 35

Figure 35. Cells probed using different anti-CNP oligonucleotide probes.

(A) Oligodendrocytes have been probed using the probe CTT GTC CTT AGC TCC TGA. The messages detected lie in the cell body and also in the major processes. Unlike the messages detected by the anti-MBP probes, the CNP messages seem to be found in a greater abundance in the major processes.

(B) The oligodendrocytes have been probed using the alternative anti-CNP probe :-
ATC TTG AAG GAA AGG GAA. This figure suggests that the CNP messages are found in the cell body and also in the processes. Of particular interest is the oligodendrocyte in the centre of the figure which shows dense silver grain staining even at relatively large distances from the cell body.

Size bar = 10μm

3.5.3. SUB-CELLULAR DISTRIBUTION OF CNP MESSAGES

Fig. 35 shows the sub-cellular distribution of CNP messages as detected by use of two different anti-CNP probes.

Fig 35A shows the distribution of CNP messages in an intact oligodendrocyte on the basis of the probe :-

CTT GTC CTT AGG TCC TGA

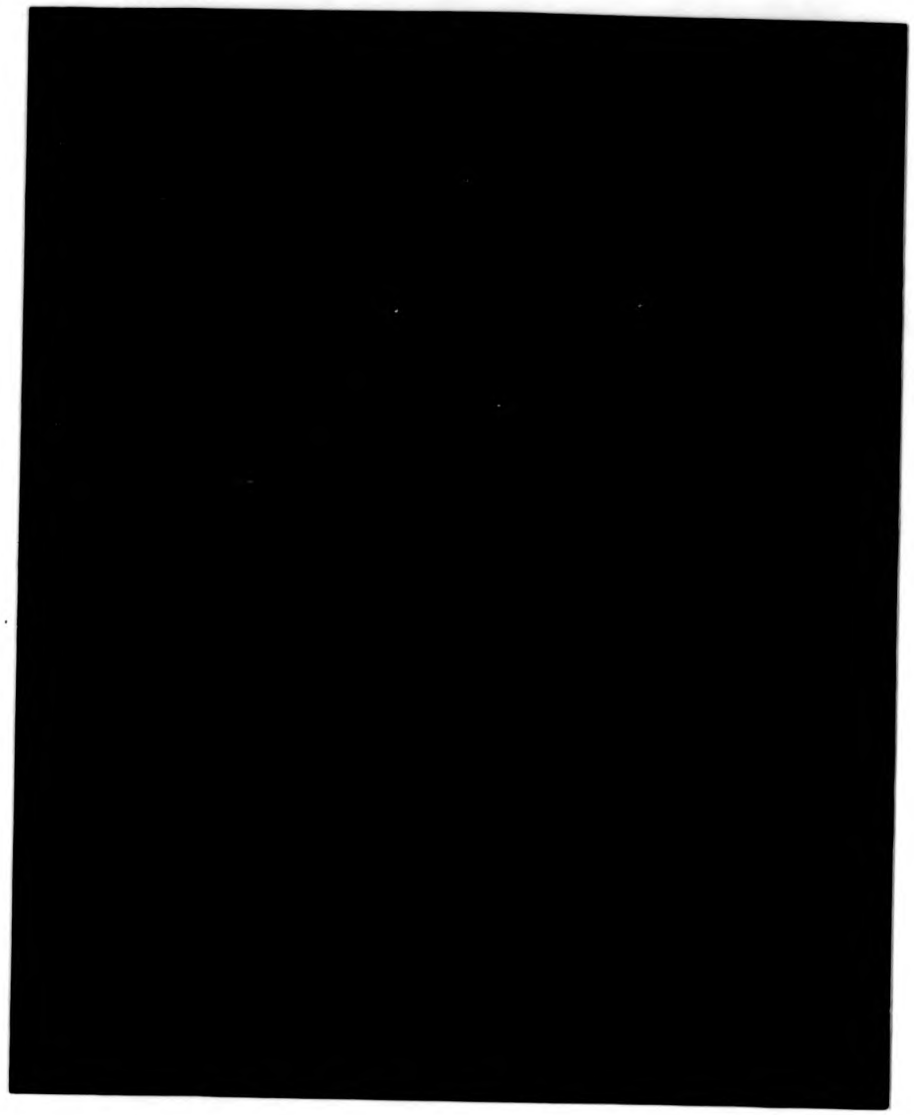
In the light of the observation made in Section 3.3.3. regarding the tendancy for CNP messages to associate with the CSK, rather than the SOL fraction, it was decided that a cytoskeletal extraction of the cell would have little effect on detection of the distribution of CNP messages, as they should be CSK associated in any event.

To check this theory and also to check for any artifactual distribution resulting from use of the probe used in Fig. 35A the probe used in Fig. 35B is an alternative anti-CNP probe:-

ATC TTG AAG GAA AGG GAA

However neither CSK extraction, nor use of a different probe results in any appreciable difference in sub-cellular detection of the CNP messages.

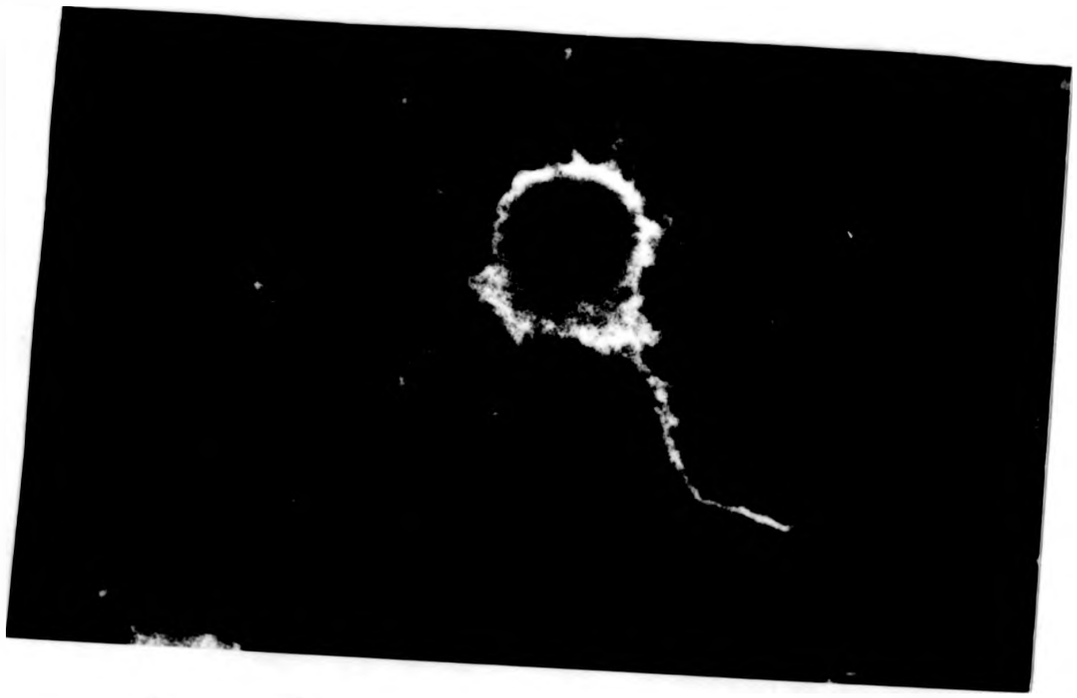
Hence the distribution pattern concluded from this work is that CNP messages tend to lie in the cell body and the major processes. This observation is in keeping with the work described by Trapp *et al* (1988) and Vogel *et al* (1988), where a similar sub-cellular distribution was reported for CNP messages in brain sections.



(A)

Figure 36

(B)



(C)

Figure 36

Figure 36. This figure shows the distribution pattern of actin messages within oligodendrocytes as detected by use of two different anti-actin oligonucleotide probes. Both (A) & (B) show the result of probing with the probe ATA GCA CAG CTT CTC TTT.

(A) The only specific staining is found in the processes of the cell in the centre of the field this is depicted by the black silver grains. In the centre of the cell is an area of brown staining which is not part of the silver grain effect. However it provides good proof that the cell has been maintained during fixation.

(B) The same pattern of actin message distribution is detected in this figure as is documented above. In this case the cells have been viewed by use of dark field microscopy, rather than bright field. Again the brown staining of the cell body offers proof that the cell has been retained intact after fixation and only the areas of white denote the existance of silver grains - these being found only in the peripheral areas of the cell.

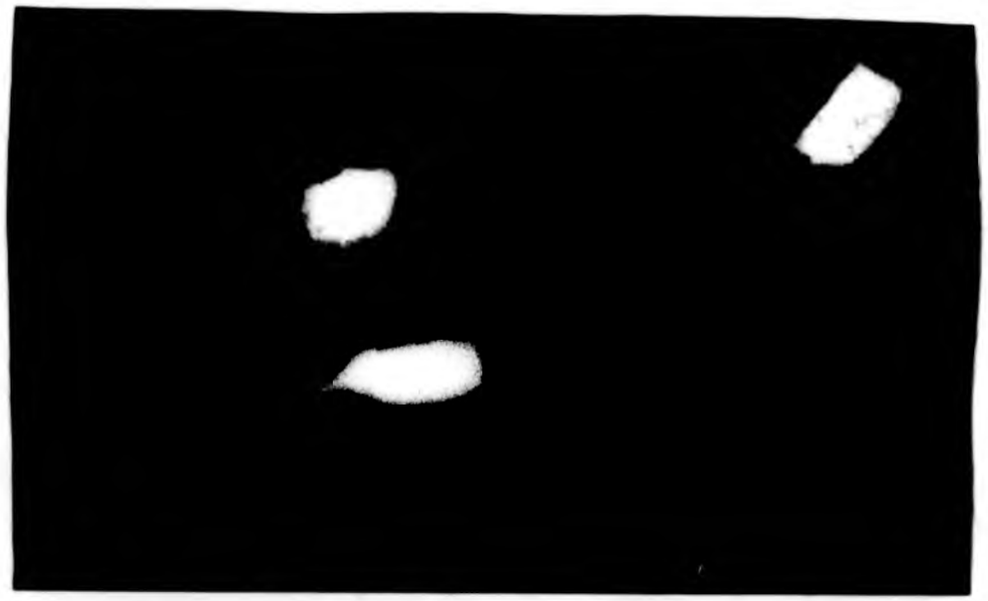
(C) This oligodendrocyte has been probed by use of the anti-actin probe TGG GGT GTT GAA GGT CTC. Again the same pattern emerges in that the only signals detected are in the very extremities of the cell body and in the processes.

It should also be noted that the probe used with success in (A) & (B) is that which failed to detect messages in human fibroblasts. Size bar = 10μm

3.5.4. SUB-CELLULAR DISTRIBUTION OF ACTIN MESSAGES

The first true sub-cellular analysis of the distribution of a specific message was performed by Lawrence and Singer (1985 and 1986) using both actin and tubulin probes.
As already described in Section 3.4.3. this work has been emulated by use of oligonucleotide probes on human fibroblasts to demonstrate here the point that oligonucleotide probes can readily substitute for cDNA probes.
In Fig. 36 the distribution of actin messages within oligodendrocytes is investigated using the two probes already described in Section 3.4.3. In all three cases the silver grains appear to lie in the more distal areas of the cell- away from the cell body. In addition it should be noted here that the probe ATA GCA CAG CTT CTC TTT which failed to hybridize to human actin messages is capable of hybridizing to rat actin messages (Fig. 36 A & B). In addition the probe TGG GGT GTT GAA GGT CTC, which hybridized to human actin messages, also hybridizes (Fig. 36C). Both probes show a tendency to hybridize to messages in the more distal regions of the cell.
This is the first analysis of the sub-cellular distribution of actin messages within oligodendrocytes. However the results were as predicted on the evidence of the actin message distribution observed in human fibroblasts (Lawrence and Singer, 1986).

(A)



(B)

Figure 37

Figure 37. As already demonstrated in Figs. 24,25 & 27 the messages encoding tubulin in oligodendrocytes lies in the cell body and to a certain extent in the major processes.

(A) The point made above is once again repeated by use of the anti-tubulin oligonucleotide probe: CCC ACC AAA GCT GTC G. The messages are again restricted to the cell body and also to the major processes, as detected by use of dark field microscopy.

(B) The alternative anti-tubulin probe : AAC ATT CAG GGC AGC GAT has been used to determine the sub-cellular distribution of tubulin messages. However there is no real difference detected by use of the alternative anti-tubulin probe, as again the messages detected are found in the cell body and to some extent in the major processes around the cell body.

Size bar = 10μm.

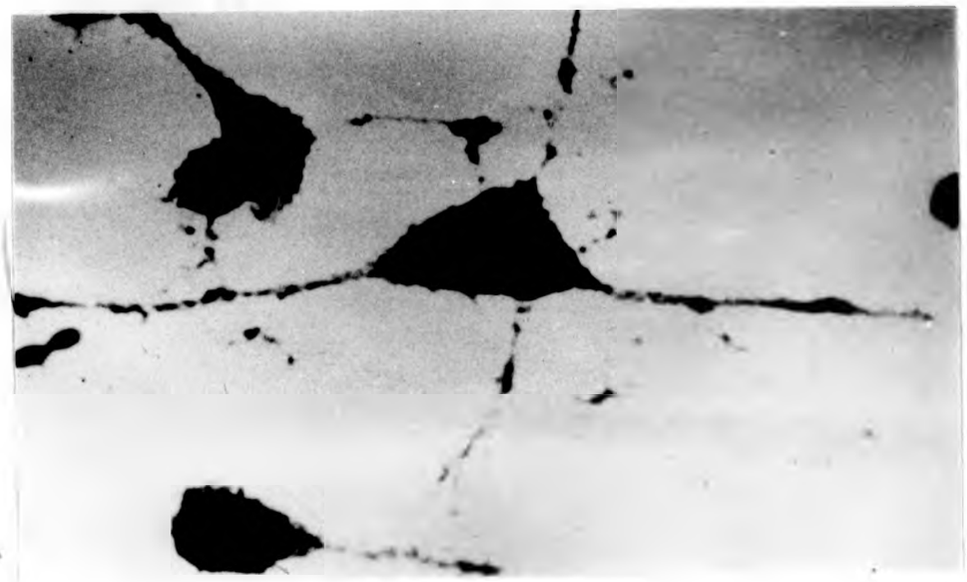
3.5.5. SUB-CELLULAR DISTRIBUTION OF TUBULIN MESSAGES

As already observed in Figs. 24, 25 & 27, tubulin messages in oligodendrocytes tend to lie in the cell body and in the larger processes around the cell body. In addition the level of tubulin message found in oligodendrocytes seems to be much greater than in other cells grown on the same coverslip, such as astrocytes. In the three examples mentioned above the probe - CCC ACC AAA GCT GTC G - was used to detect the messages.

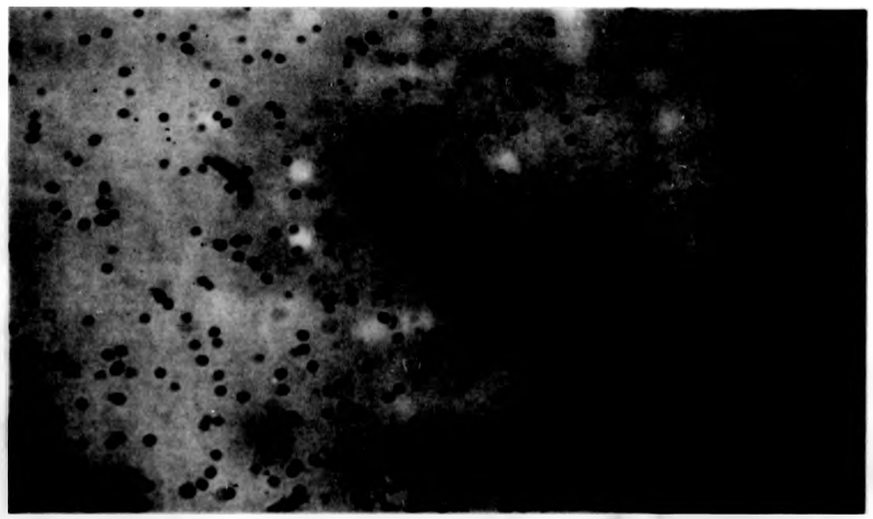
In Fig. 37A this probe is again used to locate tubulin messages. However in Fig. 37B the probe used instead was - AAC ATT CAG GGC AGC GAT. In both cases the distribution of the messages encoding tubulin is restricted to areas around the cell body, and the major processes.

The tubulin message distribution, like that of actin, has not previously been determined in oligodendrocytes. However in human fibroblasts Lawrence and Singer (1986) reported some degree of preference for localization of tubulin messages within the cell's central area. Hence the results produced here are in keeping with the overall trend observed by Lawrence and Singer (1986).

As a further note it is worthwhile commenting on the relatively lower abundance of tubulin messages within non-oligodendroglial cells. This is an interesting observation in the light of the results reported in Wilson and Brophy (1989) where it was observed that the abundance of tubulin protein in astrocytes was relatively less than that of oligodendrocytes. Hence the observation of lower tubulin message concentrations in these cells helps to explain the lower level of tubulin protein.



(A)



(B)

Figure 38

Figure 38. This figure shows cells which have been probed by use of a probe which will specifically detect messages encoding PLP, and will fail to detect messages encoding DM-20.

(A) In this figure the cell had a certain amount of brown staining in the processes, which as discussed earlier is not a positive signal. This figure demonstrates the problem of using such cells for photography with black and white film. The most dense area of staining even on the black and white picture is that of the cell body. The grey colouring of the processes is merely a reflection of the brown staining being detected. Evidence of this can be shown by imagining the nett effect of photographing Fig. 26B in black and white. Here again the brown processes would simply appear as areas of light grey.

(B) The localization of PLP encoding messages in the cell body is once again reiterated by this figure, where it is clear that the areas of most dense silver grain staining is the area around the cell body.

Size bar = 10μm

3.5.6. SUB-CELLULAR DISTRIBUTION OF PLP AND DM-20 MESSAGES

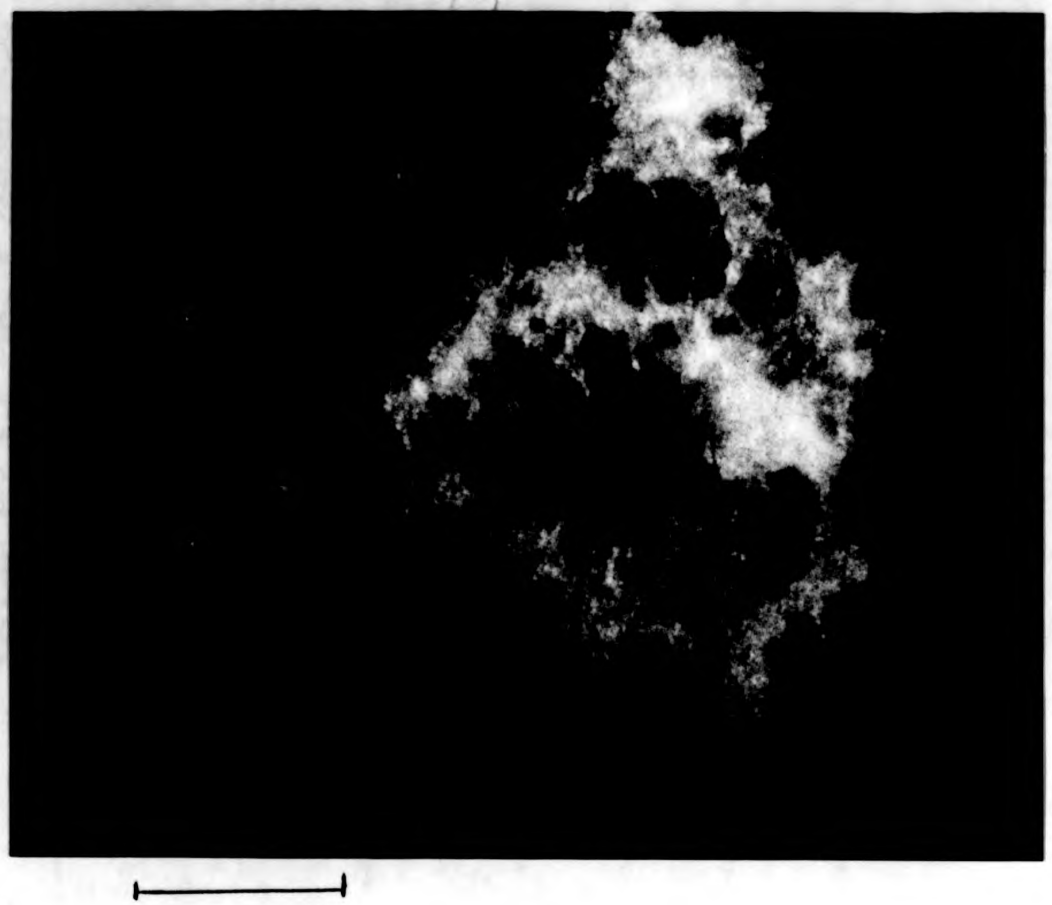
Figs. 26B and 26C have already indicated that PLP messages within oligodendrocytes tend to lie in the cell body. This point is further enforced by Fig. 38 where again the messages detected lie in the cell body.

This observation is in keeping with the results already published by Trapp et al (1987); Jordan et al (1989) and Shiota et al (1989).

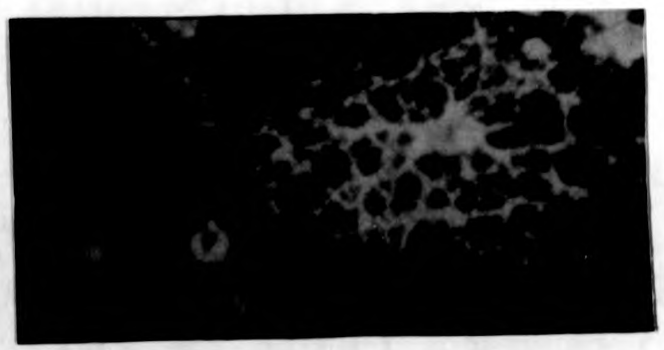
As already discussed in Section 1.3.3. the gene which encodes PLP also encodes another protein; DM-20. These two products arise from the same gene by the process of differential splicing. Since only two products arise from this gene it was hoped that an oligonucleotide might be synthesized which is specific to each message (as described in Section 1.3.3.). It was hoped that the probe synthesized to detect DM-20 would not also detect PLP, due to the intervening 105 nucleotides in the PLP message. Assuming this to be the case it was proposed to carefully study the in situ hybridization results generated by the two different probes to search for any subtle differences between the two.

Fig. 39 demonstrates the distribution of messages detected by the DM-20 specific probe. Quite unexpectedly the difference between PLP and DM-20 messages, as regards sub-cellular distribution, is on a major scale, rather than the minor difference which had been expected at most. Instead of being located in the cell body the DM-20 messages appear to lie in the very most distant areas of the cell, being almost excluded from the cell body.

In addition /...

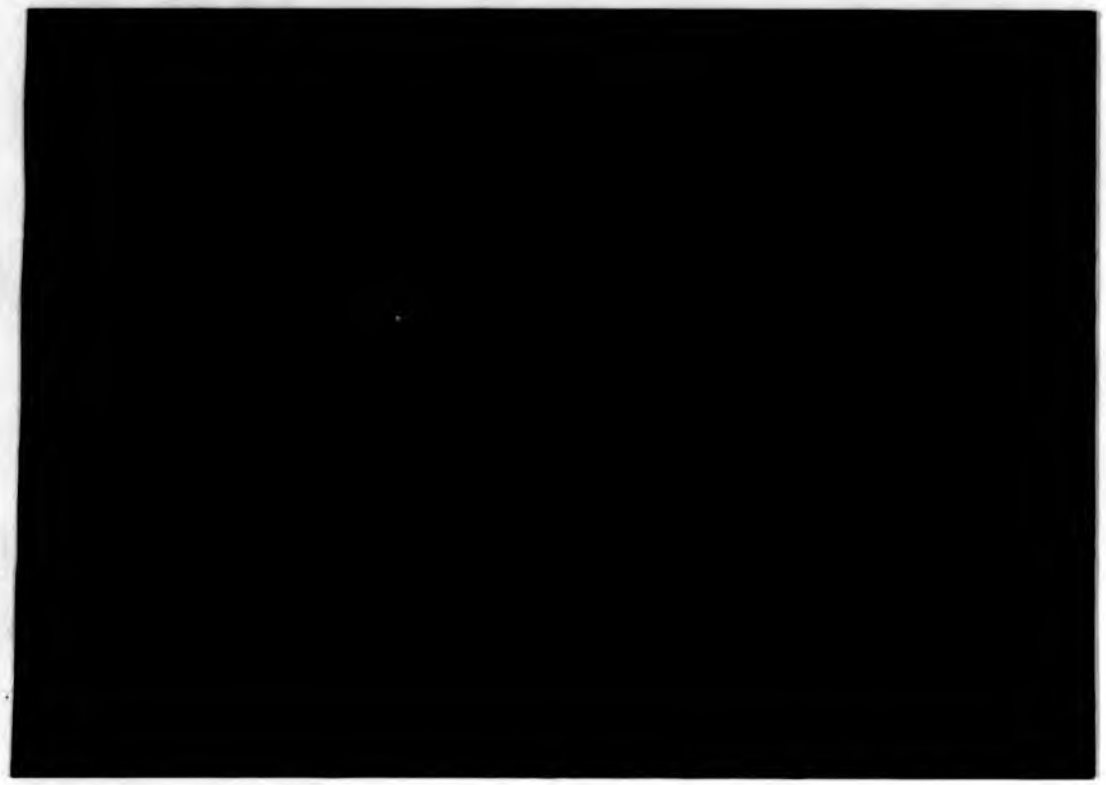


(A)

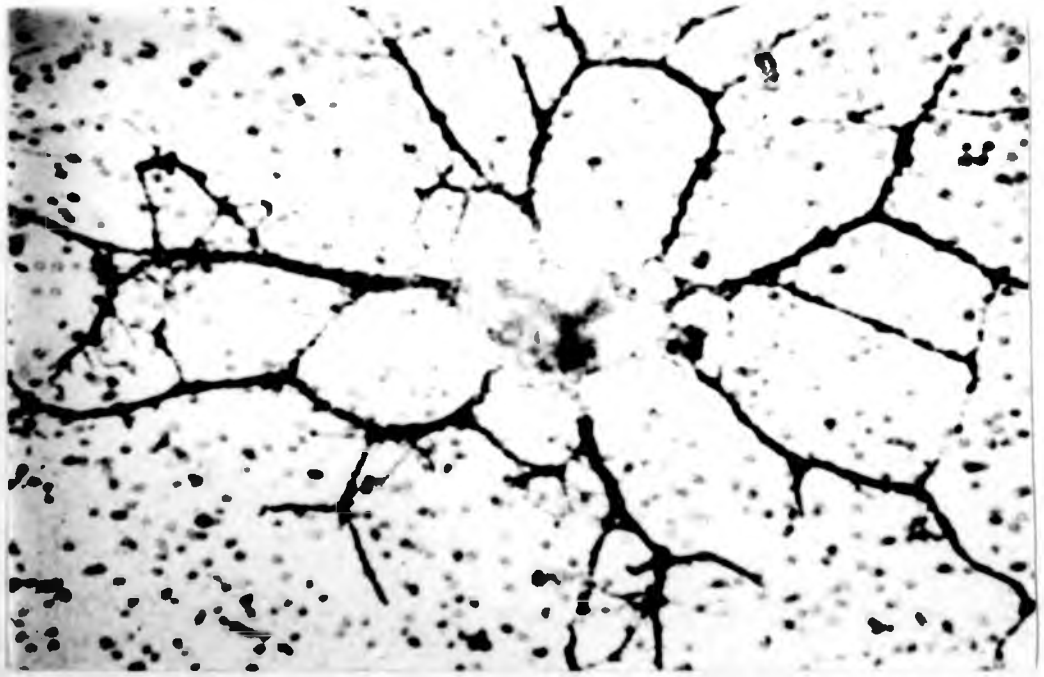


(B)

Figure 39



(C)



(D)

Figure 39

Figure 39. Both (A) and (B) show oligodondrocytes which have been probed for their DM-20 encoding messages. By use of dark field microscopy these messages have been localised to the most peripheral areas of the oligodendrocyte cell - the processes. This is in sharp contrast to the observations made for the messages encoding PLP. It is all the more interesting to remember that these two types of message are the product of a single gene.
(C) & (D) are photographs of a single cell. In (C) the oligodendrocyte has been photographed by use of bright field microscopy. Quite clearly the majority of silver grains lie over the processes. In the centre of the cell there is a mass of brown staining, which as already discussed is not a positive signal. (D) shows the same cell. In this case the cell has been processed onto a photographic slide. This coloured slide was then rephotographed against a brown background filter and in so doing the resulting black and white photograph shows only the areas which had been detected by observing silver grains. Quite clearly this area is the cell processes.
Size bar = 10μm.

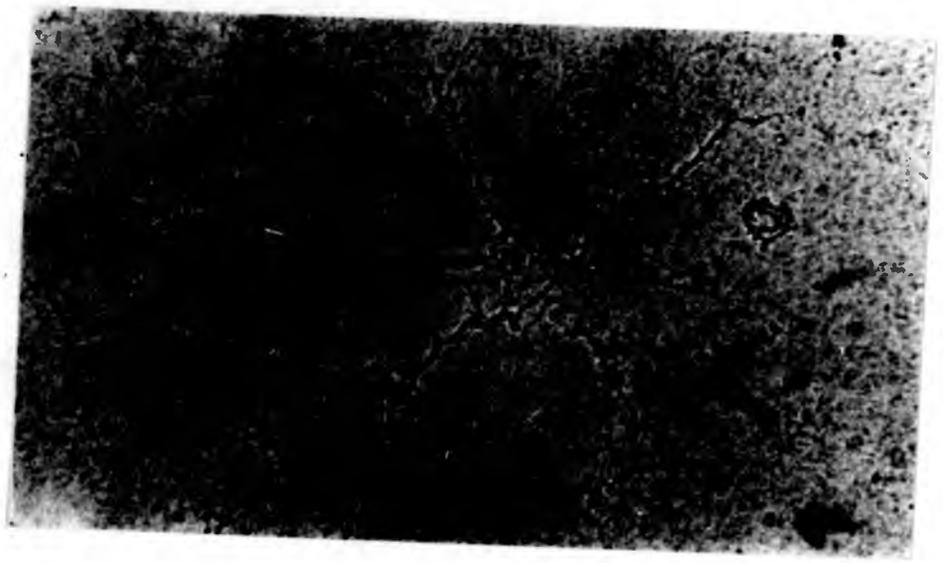


Figure 40

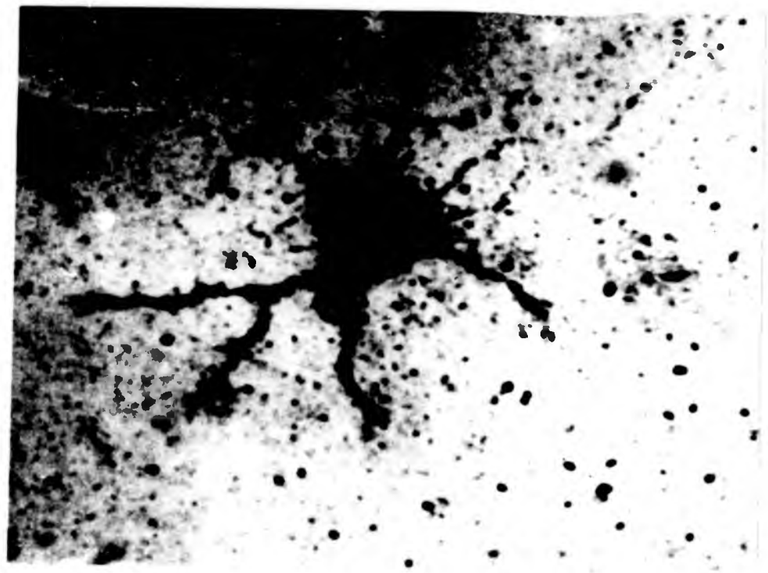
Figure 40. This figure shows the result of probing the cytoskeletal remains of an oligo dendrocyte with the probe designed to specifically detect DM-20 encoding messages. Quite clearly a reasonable amount of the DM-20 message remains in the CSK residue. This is in keeping with the observations made earlier in the thesis using dot blots on RNA which had been extracted from cells and dotted onto nitrocellulose. In addition the pattern observed in the intact cell, as regards the distribution of DM-20 messages is continued in this figure - the messages lie in the processes of the cell.
Size bar = 10µm.

In addition to the results in Fig. 39 using whole cells, the CSK residue of an oligodendrocyte was also probed for DM-20 distribution (Fig.40). Again the same trend emerged; the DM-20 messages are found in the more peripheral areas of the cell.

Unlike the other messages where use of a second probe is possible to confirm the observations being made regarding message distribution it is not possible to use an alternative DM-20 specific probe. This is a reflection of the fact that only a small sequence of the DM-20 message may be probed against. Instead it was decided to synthesize a probe which has the capacity to hybridize to messages encoding either PLP or DM-20. Use of such a probe should allow detection of all products of the PLP gene.

From Fig. 41 it is clear that the probe has the capacity to hybridize to messages in both the cell body and the cell's processes. Hence it would appear that the results observed with the DM-20 probe are genuine.

In addition the fact that cells show distribution of PLP/DM-20 throughout the cell suggests that oligodendrocytes carry both messages, and that they are not either PLP-specific or DM-20-specific cell types.



(A)

(B)

Figure 41

Figure 41. (A) shows an oligodendrocyte which has been probed for the distribution of both its PLP and DM-20 encoding genes by use of an oligonucleotide probe which has the capacity to detect both messages. Quite clearly there is a considerable amount of message detected in the cell body, but there is also a large amount of messages detected in the processes around the cell body. This suggests that both the PLP messages in the cell body and the DM-20 messages in the processes are being detected by this oligonucleotide probe.
The observation that both messages are being detected in a single cell is interesting as it means that there is not one pool of cells which are specific for PLP and another set of cells which are specific for DM-20 messages.
It is also worth comparing the processes of this cell where there is a positive signal and the of the cell in Fig.38A where there is only the brown staining present. Clearly in this cell the processes are black rather than the faint grey colour observed in the negative region of Fig.38A (B) The cells seen in this figure again reiterate the comments made for (A), as messages are detected in both the cell body and the processes when using a probe capable of detecting both PLP and DM-20.
Size bar = 10μm

(A)

(B)

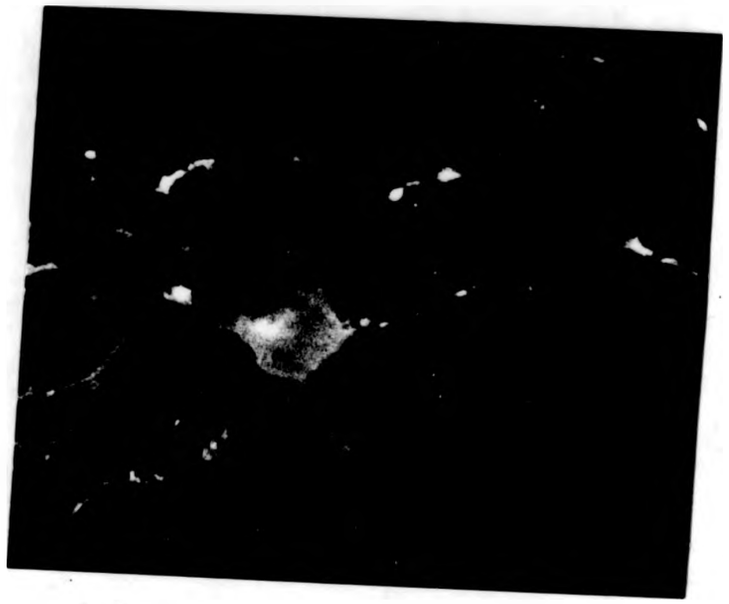
Figure 42

Figure 42. This figure shows joint <u>in situ</u> hybridization and immunocytochemistry being performed on a single cell.

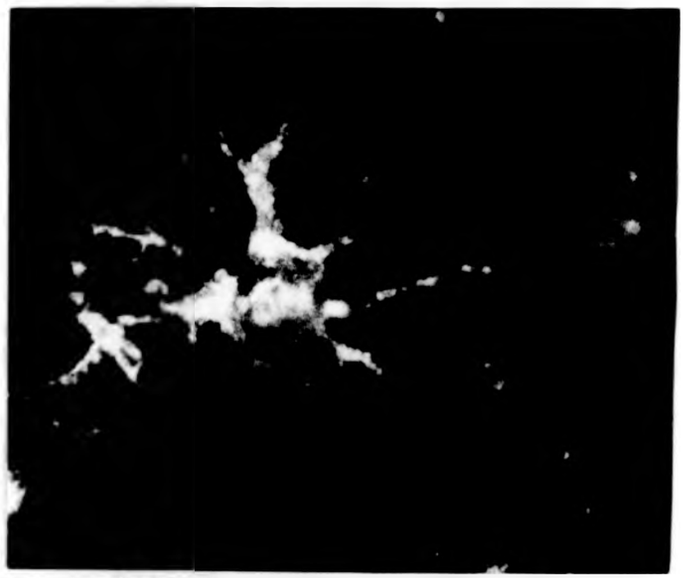
(A) The cell has been probed for its content of MBP proteins by use of a rabbit-anti-MBP antibody. In turn this antibody was detected by use of an anti-rabbit antibody which has been labeled with a fluorescein marker. This fluorescein marker was detected by use of a fluorescent microscope. Hence by a process of indirect fluorescence the distribution of the MBPs can be determined. In this cell the MBPs lie largely in the cell body and in the processes closest to the cell body, although a certain amount of the protein is also found in the more distal processes.

(B) The MBP message distribution of this cell has been detected by use of <u>in situ</u> hybridization. Like its protein counter-part the messages encoding MBP lie in the cell body and the major processes. However in the case of the MBP messages nothing is found in the more distant processes. Nevertheless there is a great deal of co-localization observed between the MBPs and their corresponding messages.

Size bar = 10μm.



(A)



(B)

Figure 43

Figure 43. This figure shows a comparison of the sub-cellular distribution of CNP and its corresponding messages within an oligodendrocyte.
(A) shows the distribution of CNP proteins as determined by use of indirect immunofluorescence. Quite clearly CNP is found throughout the entire oligodendrocyte cell. It is possible that there is a majority of the protein found in the cell body and the surrounding major processes. However there is also a considerable quantity of the protein found in the finer processes of the cell.
(B) The CNP encoding messages are found in the cell body, but also to a large extent in the major processes. However unlike the protein it encodes, the CNP message is unlikely to be found in the minor processes.
Hence it seems that there is some degree of co-localization between the CNP protein and the CNP message within an oligodendrocyte. However at least some of the protein must be synthesized in a more central area of the cell and only then transported to the most extreme areas of the cell.
Size bar = 10μm.

3.5.7. JOINT in situ HYBRIDIZATION AND IMMUNOCYTOCHEMICAL EXPERIMENTS

The overall trend observed so far is that messages and their corresponding proteins have a tendency to show some degre of co-localization, with the possible exception of DM-20. This observation is based upon the results generated here for in situ hybridization and the results presented in Wilson and Brophy (1989) for immunocytochemistry.

To illustrate this co-localization point better it was felt that the use of both techniques in a single cell would be preferable to comparing results from two different cells, one technique being applied to each.

Development of the technique to allow joint immunocyto-chemistry and in situ hybridization to be applied to a single cell is described in Section 3.4.2. The results presented in Figs. 42-48 illustrate this point, again the possible exception being DM-20, although the presence of a protein in the processes of the cell which is recognised by the PLP/DM-20 antibody is slightly stronger than was reported in Wilson and Brophy (1989).

Fig. 42 demonstrates that the majority of the MBP messages are found primarily in the cell body, with a certain amount in the major processes, and that there is a degree more of the protein found in the processes.

Fig. 43 demonstrates that the majority of the CNP messages are in, and around, the cell body, although substantial amounts are in the major processes. This pattern is generally reflected in the protein distribution although the CNP protein is also found in the minor processes.

Fig. 44 /...

(A)

(B)

Figure 44

Figure 44. This figure demonstrates the high level of co-localization observed between actin encoding messages and their protein counter-part.

(A) Filamentous actin has been detected by use of Rhodamine Phalloidin, which in turn was observed by fluorescent microscopy.

Clearly all the protein being detected lies in the processes of this cell, with staining in the cell body being absent.

(B) The actin encoding messages have been detected by *in situ* hybridization using an oligonucleotide probe. The same distribution pattern is observed for the messages as was observed for the protein itself - the actin messages all lie in the processes of the cell.

This particular example of protein and message co-localizing is probably the most striking of all presented in this thesis.

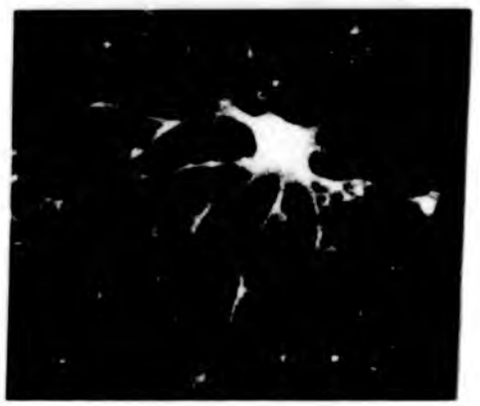
Size bar = 10μm.

Fig. 44 demonstrates what is probably the most extreme form of co-localization of protein and message. The actin message and the filamentous actin are found co-localized in the processes of the cell and appear to be completely absent from the cell body.

Fig. 45 demonstrates that the tubulin message is generally found in the centre of the intact cell and also in the centre of the CSK residue of an oligodendrocyte. In a similar manner this too is observed for the tubulin protein in both the intact cells and the CSK residue, although a certain amount of the tubulin protein was also found in the processes of the cells.

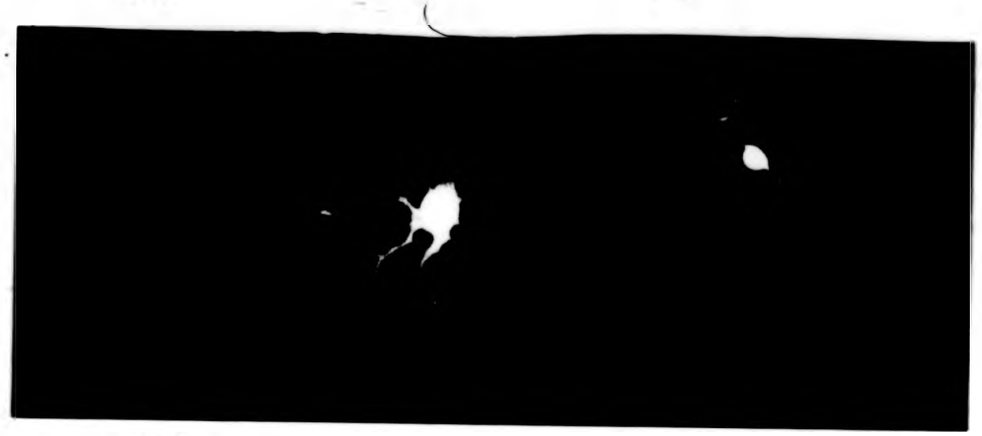
Figs. 46-48 probably present the most interesting story as Figs. 46A, 47A and 48A all suggest that the PLP/DM-20 proteins have a strong tendency to lie in the cell body, with only a small amount found in the processes. As might be expected, Fig 46B shows that the PLP encoding messages lie in the cell body. However like Fig 40, Fig. 47B suggests that the DM-20 messages lie in the more distal positions of the cell, and from a comparison with Fig. 48B this point is reiterated. No obvious explanation for this phenomenon can be presented, although possible causes are discussed in Section 4.

In conclusion it appears that messages have a tendency to co-localize with their protein counter-part, although in most instances the proteins have a tendency to be found slightly more distally than the messages.



(A)

(B)



(C)

(D)

Figure 45

Figure 45. This figure demonstrates the high level of co-localization observed between tubulin and the corresponding message.

(A) This cell has been probed by immunocytochemistry to detect the tubulin distribution by indirect immunofluorescence. Clearly most of the tubulin detected is found in the cell body, with a certain amount found in the major processes.

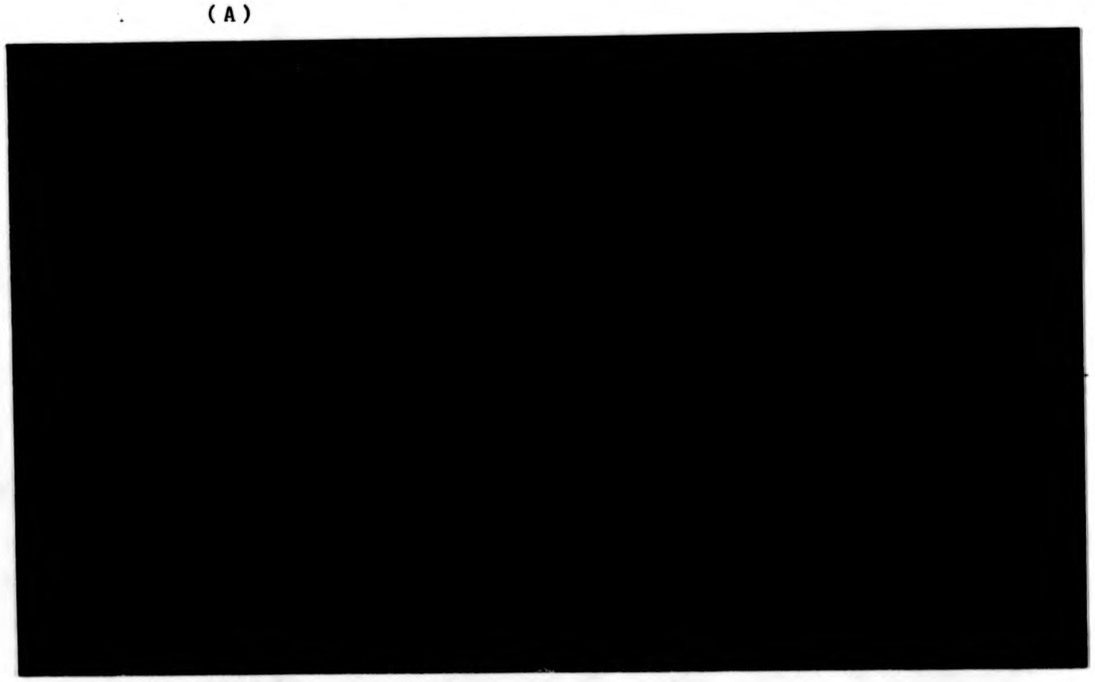
(B) The same cell as described in (A) was probed for its tubulin message distribution by use of in situ hybridization using the oligonucleotide probe : AAC ATT CAG GGC AGC GAT. The messages are all found in the central area of the cell.

(C) Shows two cells probed for their tubulin protein distribution by means of immunocytochemistry as described in (A). Again the same pattern is seen - the protein lies mainly in the cell body, but a certain amount is also found in the major processes, and a trace amount in the minor processes.

(D) is the same field as described in (C) but here the cells have been probed for their tubulin message distribution by in situ hybridization using the probe : CCC ACC AAA GCT GTG G. The same pattern of distribution is observed as was observed in (B) using the alternative anti-tubulin probe :- messages lie in the cell body with a small amount in the major processes.

Size bar = 10μm.

(A)



(B)

Figure 46

Figure 46. (A) The PLP/DM-20 distribution of an oligodendrocyte. The distribution is detected by use of indirect immunofluorescence using an antibody which is able to detect both proteins. The bulk of the signal being detected is found in the cell body, although a small amount is also detected in the processes.

(B) The PLP encoding messages are detected by use of in situ hybridization using an oligonucleotide probe designed to specifically detect PLP messages. Here the messages detected are confined to the cell body.

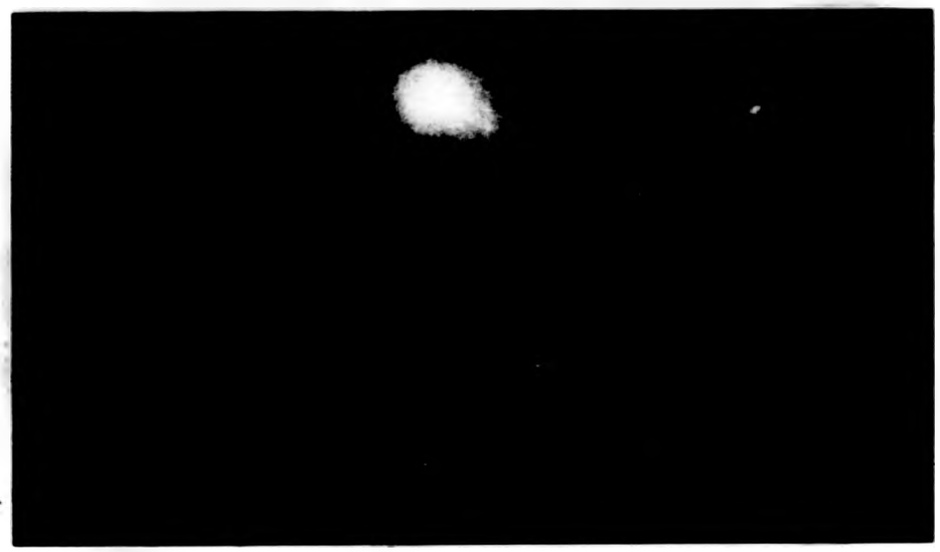
Size bar = 10μm

(A)

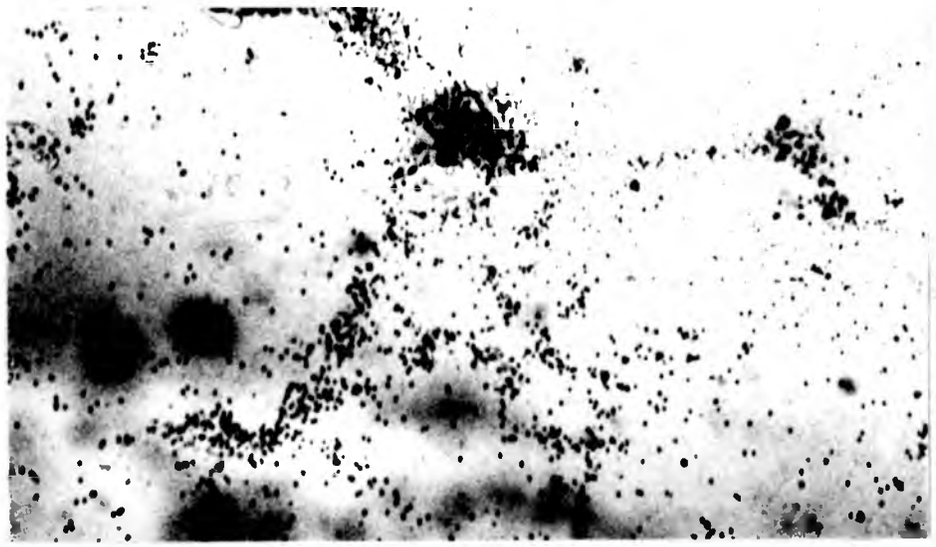
(B)

Figure 47

Figure 47. (A) The PLP/DM-20 protein distribution in an oligodendrocyte as detected by use of immuno-cytochemistry and indirect immunofluorescence. The majority of the protein being detected lies in the cell body with only a small amount lying in the processes of the cell.
(B) The DM-20 encoding messages are detected by use of in situ hybridization using an oligonucleotide probe designed to specifically detect DM-20 messages. In this case the messages being detected lie in the processes, with only trace amounts in the cell body.
Size bar = 10μm.



(A)



(B)

Figure 48

Figure 48. (A) The PLP/DM-20 protein distribution within an oligodendrocyte as detected by use of immunocytochemistry and indirect immunofluorescence. The bulk of the protein is found in the cell body with a small amount found in the processes.
(B) The PLP/DM-20 message distribution as detected by *in situ* hybridization for the cell described in (A). The messages are detected by use of an oligonucleotide which has the capacity to detect both messages. Like the pattern observed for the protein distribution there is a strong signal from the cell body. However unlike the faint signal detected for the processes with the antibody there is a strong signal detected in the processes when *in situ* hybridization is used.
Size bar = 10μm.

CHAPTER FOUR

DISCUSSION

4. DISCUSSION

Before discussing any other results generated in this thesis it is important to assess the meaning of the Northern blot studies from Section 3.2.2. In order that the probes used in later experiments may be used with some degree of confidence it is vital to establish that they are specific for the messages they were intended to detect. The only way to prove this specificity is to use Northern blots.

As already discussed, Northern blots do not actually prove that only one species of message is being detected. Instead these results show that only messages of one particular size are being detected. Hence in theory it is possible that a number of different messages are being detected, and that they are all of a similar size. In reality this is not very likely, but it is nevertheless a possibility which must be borne in mind. For example the probes used to detect the MBP messages only detect a single band on an autoradiogram, but it is already known that this single band actually comprises the detection of four, or more, different messages. However in the absence of any better method of proving the specificity of the probes for a single message type Northern blots will have to suffice.

It is also worthwhile commenting on the build-up of the Northern blot hybridization method. While a number of methods of hybridization were tried, it is possible that some of these would have generated results identical to those eventually obtained, if the 0.1μm pore nitrocellulose filter had been used in the first place. It was felt that little could be gained from repeating these methods with the alternative filter as the point which had been sought in the first place had already been /...

been proven - that only messages of a certain size are being detected, based upon size fractionation on an agarose gel. It may be possible to improve upon Northern blots as a means of showing specificity to one species of RNA by means of the recently marketed magnetic beads from Dynal. It should be possible to bind some of the oligonucleotide probe to the magnetic beads. Hybridization would then be performed between the oligonucleotide bound to the bead and the RNA sample normally run on the gel. By applying a magnet to the hybridization tube, it is possible to wash away the remaining RNA, leaving only that which is specifically bound to the probe. The next stage in this process would be to heat the tube to denature the oligonucleotide-RNA hybrid, hence allowing the messages which had specifically bound to the probe to be isolated. These messages may be translated in vitro and the resulting proteins run on a polyacrylamide gel. Since it is possible to run 2-D protein gels in a manner not available for RNA gels, it may be possible to detect a single spot or band on a protein gel and so increase the level of confidence with which single message detection may be declared. Unfortunately the idea of using magnetic beads in conjunction with in vitro translation is only a hypothesis which has not yet been successfully reported, and indeed may not even have been tried. The major problem that might be encountered in this proposed method is that recovery levels of RNA are likely to be very low, which makes recovery by ethanol precipitation difficult. The second problem in the method is that the RNA which might be isolated must still be capable of translation, which, due to the number of manipulations being performed, is not a foregone /...

not a foregone conclusion.

However until such a method is devised Northern blots remain the best method for demonstration of specificity. In the present work it might be argued that RNA material from an alternative source other than rat brain should have been used to highlight the specificity of the probes. This is a valid point in so much as it should lead to a totally blank lane running alongside a lane which contains a single band on an autoradiogram.

Exactly what type of RNA to use for negative controls is a matter for debate. Use of RNA from a source other than brain is one possibility (e.g. rat liver). However other than demonstrating the absence of the detected message from this particular tissue there is little to be said for this as being much advantageous over the nucleic acid components of the pre-hybridization buffer. Instead it would be better to use RNA extracted from brain tissue of another species. The problem with this approach is that there are relatively few sequences derived for cDNAs from myelin genes. Nevertheless sequences for murine MBP (de Ferra et al, 1985), bovine CNP (Vogel and Thompson, 1987) and human PLP/DM-20 (Simons et al, 1987) are already available. However the probes selected and synthesized are often capable of hybridization to other species due to the high level of sequence conservation. For example the MBP probes reported here are 100% complementary to both rat and mouse messages, and the DM-20 probe is 100% complementary to both rat and human messages. There is a single mis-match for the PLP probe between rat and human messages, so it should have been possible to do studies with these two messages, /...

messages, but in the case of the DM-20 probe it is impossible to use any alternative probe as only this small target sequence is available.

Hence to use probes which are sufficiently different from one species to another it is essential to closely study the cDNA sequences of two species. This is one method of selecting probes, but as already documented the method of choice for probe selection was based upon obtaining a suitable tail for terminal transferase labeling of oligonucleotide probes (see Section 2.3.1.).

Hence the probes for hybridization studies have been found to be acceptable for the work described, as defined by the requirements they were designed to meet before they were synthesized.

The next important issue to examine is the source of material for *in situ* hybridization studies. Two forms of oligodendrocytes may be studied; those grown *in vivo* and those grown *in vitro*. Obviously cells grown *in vivo* are more representative of the oligodendrocyte as it developed in the brain. However for the purpose of sub-cellular studies it is unlikely that a transverse section of the rat brain will produce a cross-section of any oligodendrocyte in a single plane.

As already detailed in Section 2.1.1., correct oligodendrocyte differentiation is dependent upon signals from brain cells other than other oligodendrocytes. Evidence exists that astrocytes play an important role in this governing of correct differentiation (Bhat *et al*, 1981). In addition it appears difficult to believe that axons are not involved in this process too, as these are the very cells which are to be myelinated /...

myelinated by the developing oligodendrocyte.

The work presented here involves in situ analysis performed on tissue culture grown cells. Since the oligodendrocytes were grown in the absence of the cells with which they normally interact and myelinate, it is clear that they were being grown in an artificial environment. Therefore choosing cells from such a source requires some justification.

The presence of the astrocytes in the culture flasks allows the oligodendrocytes to be exposed to the astrocytic signals required for proper differentiation. This just leaves the question of the axons, which are absent. However in the light of the results presented here, relative to that published for sections, the problem seems to be of a minor nature. Nevertheless there is one fundamental advantage to be obtained from the use of tissue culture methods. This advantage is reliant on the morphology of the oligodendrocytes. These cells have such a complex morphology that to cut the cell in a single plane is to all extents and purposes impossible. Therefore the only way to view an entire oligodendrocyte cell is to use cells grown in tissue culture, albeit in the absence of axons. The messages investigated at the sub-cellular level in oligodendrocytes have been discussed in Section 1.4.6. and it is clear that in all cases the signals tend to be distributed in small massed groups - indicative of messages found around the cell body of the oligodendrocyte. Given the limitations of cells cut in section, it is difficult to believe that the messages which are found in the more peripheral areas of the cell (e.g. DM-20 and actin encoding) could have had their sub-cellular distribution as readily detected. Indeed it is likely that the signals derived from in situ hybridization performed /...

performed on these messages when viewed in brain sections, would appear as either non-specific binding or at best as a number of different lines, which represent the processes of the cells. Hence tissue culture techniques allow for a more refined analysis of the sub-cellular distribution of messages. It is interesting to note that those messages which had previously been studied by use of sections of the brain show the same sub-cellular distribution as found here with cultured cells. This of course does not prove that the absence of axons is of little consequence, but on reflection there appears to be insufficient differences between the *in situ* hybridization results presented here and those presented elsewhere. In addition the previous results only deal with messages found in the cell body. However from Lawrence and Singer (1986) it was expected that the actin message would be peripheral. Possibly the only way to address this problem would be to perform *in situ* hybridization experiments on myelinated axons at the electron-microscope level, as the most distal regions of the oligodendrocyte will now be wrapped around the axons and only at this level of microscopy can the messages be sought with any degree of refined accuracy.

Having justified the parameters for investigating the subject of sub-cellular distribution, it is worthwhile dealing with the type of site of translation used by the messages as regards free/bound polysomes or cytoskeletal/non-cytoskeletal association. It is already documented that CNP and MBP messages are translated on free polysomes (Gillespie *et al*, 1990a; Colman *et al*, 1982) and that PLP is translated on bound polysomes. From the knowledge that the translation of PLP messages is performed on bound polysomes /...

polysomes, it has been proposed that the DM-20 messages are also translated on bound polysomes, since these two messages are the product of a single gene.

The dot blots used to analyse the distribution of messages between the cytoskeleton-bound and the soluble fractions were performed using the detergent extraction proceedures described in Gillespie et al (1989) and Wilson and Brophy (1989) who have already described the build-up of the methodology for this segregation into the two pools.

None of the six messages investigated was found exclusively in the non-cytoskeletal pool of messages, but actin, tubulin, and CNP messages were found almost exclusively in association with the cytoskeleton. In addition messages encoding MBP, PLP and DM-20 are found in both pools. This supports the observation of Terasaki et al (1986) when they found that messages translated on the rough endoplasmic reticulum may be associated indirectly with the cytoskeleton.

It is interesting to note that Fey et al (1986) found that free polysomal messages in HeLa cells must be associated with the cytoskeleton in order that they may be translated. However despite the fact that MBP messages are translated on free polysomes, there are still MBP messages to be found free of the cytoskeleton. In addition these messages found in the soluble fraction are still capable of translation in vitro using wheat germ extract (Gillespie, 1988).

This suggests that the coding region of the MBP message is still intact, although there is a possibility that degradation has occurred outwith the coding region. Assuming Fey's observation holds true for MBP messages, requiring cytoskeletal association for translation to occur, then either the MBP messages /...

messages found in the soluble pool have lost their *in vivo* capacity for translation or in some way this capacity is being masked.

The exact reason for this distribution of the MBP messages between the two pools is unclear, although it is interesting to note what appears to be a difference in the sub-cellular distribution of MBP encoding messages between the CSK fraction and the total RNA pool, on the basis of *in situ* hybridization (Section 3.5.1.). From this it may be inferred that there is a difference in the sub-cellular distribution of the MBP messages within the two pools; CSK and SOL.

In addition it is interesting to note that Gillespie (1988) documented that on the basis of *in vitro* translations there was a tendency for the 21.5 kDa encoding messages to be found in association with the CSK. This begs the question of a difference in sub-cellular distribution of the messages encoding the various differentially spliced products from the MBP gene. This has been tackled to some degree by Staugaitis *et al* (1990) where the HeLa cells were transformed using mouse cDNAs for 4 MBPs; 14, 17, 18.5, 21.5 kDa. Cells transformed with either 14kDa or 18.5kDa encoding DNAs produced messages which migrated to the peripheral region of the cell, which implies a close association with the membrane. Hence they inferred that the 14kDa and 18.5kDa MBPs have a strong, non-specific membrane-biding properties which mediate compaction of the membrane sheaths which form myelin. Furthermore it has been suggested that the 17kDa and 21.5kDa MBPs have a role to play in the early initiation processes between cells before the onset of full myelination (Kamholz *et al*, 1986). The distribution /...

distribution of messages relative to age is also worthy of comment, as the 14kDa and 18.5kDa MBPs are proportionally more abundant in more mature animals (Barbarese *et al*, 1978). The results presented by Staugaitis *et al* (1990) were of course achieved using HeLa cells which is an artificial environment. Probably there is only one method to study the distribution of the MBP encoding messages. Unlike PLP and DM-20 where only 2 messages require study, the presence of four (or more) such messages complicates the issue. For the present only 4 MBP messages have been reported in rats, which is the study for the work presented here. Therefore the method described below is based upon the assumption that there are only 4 types of message to be detected, which in truth may be an error.

To investigate the task described above 4 oligonucleotides must be synthesized;

i) an oligonucleotide specific for exon II

ii) an oligonucleotide specific for exon VI

iii) an oligonucleotide which bridges the 3' nucleotides of exon I and the 5' nucleotides of exon III

iv) an oligonucleotide which bridges the 3' nucleotides of exon V and the 5' nucleotides of exon VII.

It is assumed that oligonucleotides (iii) and (iv) will only hybridize when exons II and VI respectively have been spliced out by differential splicing, in a manner similar to that described for the DM-20 specific oligonucleotide (see Section 3.5.6.).

Hence to detect the message encoding the 21.5kDa protein, oligonucleotides (i) and (ii) would be used in an *in situ* hybridization /...

hybridization experiment. Oligonucleotide (i) would hybridize to messages encoding both the 21.5kDa and 17kDa proteins. Oligonucleotide (ii) would hybridize to the 21.5kDa and 18.5kDa encoding messages. Treatment with a single stranded exoribonuclease which causes digestion of messages from both the 5' and 3' termini will cause only regions protected by the hybridized oligonucleotides and the intervening regions to remain intact.

This will lead to total digestion of the 14kDa encoding message, and total digestion of the 17 and 18.5kDa encoding messages (except for the small protected sequence- protection being afforded by the hybridized oligonucleotides). However the message encoding the 21.5kDa protein is only digested as far as the oligonucleotides and so exons III, IV and V all remain intact. Hence a probe directed against MBP will specifically detect the 21.5 kDa encoding message, as it is the only one which remains undigested.

The messages encoding the 14, 17 and 18.5 kDa proteins may also be detected by varying the combination of oligonucleotides selected to afford protection to the messages. The major problem in this work is that such an exonuclease has not yet been reported for RNA. However should such an enzyme become available this is most certainly an interesting avenue to study.

It is unfortunate that such work cannot currently be used for the MBP encoding messages. However the possibility of studying the distribution of PLP and DM-20 messages has already been tackled here. In Section 3.5.6. the PLP messages are shown to lie in the cell body and the DM-20 encoding messages are found /...

found in more peripheral areas of the cell. In addition using a probe capable of detecting the two messages it appears that cells investigated have both messages expressed at any one time. This is based on the observation that this probe detects messages throughout the cell, rather than just in the processes or just around the cell body, as is the case for the DM-20 and PLP specific probes. Possibly this could be improved upon by using the DM-20 specific probe and the PLP specific probe together, one labeled with a radioactive tracer and the other labeled by non-radioactive means. Such non-radioactive labels could include 5'-bromodeoxyuridine (F. Ortigao, personal communications) or biotin. The use of biotin labeling probes for work with oligodendrocytes might cause some concern due to the presence of endogenous biotin in the rat brain (LeVine and Macklin, 1988). However LeVine and Macklin found that heating the tissue to 55°C before any work was performed destroyed the capacity for avadin to detect the residual biotin and so any detected at a later stage must be due to the labeled probe.

Returning to the observation that the DM-20 encoding message is found in the peripheral area of the cell provides the question of truth in this observation. A couple of points must be tackled first before declaring the authenticity of this work. Firstly it is thought that messages translated on bound-polysomes have a tendancy to lie in the centre of cells (Terasaki et al, 1986). The messages encoding DM-20 are assumed to be translated on bound polysomes. Hence it would appear more likely that these messages should be found in the cell body, rather than in the processes. For this reason a number of requests were made for antibodies raised specifically against the rough endoplasmic /...

endoplasmic reticulum. This would allow a subcellular study of the distribution of the rough endoplasmic reticulum in oligodendrocytes and hopefully would illustrate a distribution throughout the cell, which would support the observation made regarding the DM-20 message distribution. Unfortunately no such antibodies were made available, and raising such an antibody was felt too time-consuming a task.

The second problem posed by this observation is that it appears that most DM-20 protein lies in the cell body, along with the PLP protein, as detected by immunocytochemistry. The fact that the antibody used here is capable of detecting DM-20 has already been demonstrated in Western blots (Wilson and Brophy, 1989). Hence either the DM-20 cannot be detected in vivo, the permeabilization process using 0.1% Triton X-100 leads to loss of DM-20 proteins or the messages are translated away from the site of protein localization.

The inability for detection of DM-20 would rely upon DM-20 being protected from detection by some means, such as being totally enclosed by other molecules. Probably the best method to study DM-20 specifically is to raise an antibody against a polypeptide which is composed of the last few residues encoded by exon IIIA and the first few residues encoded by exon IV (in a manner similar to that use for the DM-20 specific oligonucleotide already described). If this antibody is unable to detect anything within a cell, it must be assumed that the protein is either lost or inaccessible. However if the protein is found in the cell body, then it must be assumed that the protein and message do not co-localize.

However the current data dictates that it must be assumed that the PLP /...

the PLP and DM-20 encoding messages do not co-localize. Hence this suggests that they must be kept apart for some reason. Exactly what this reason is, is currently unclear, but it is worth noting that a difference has been detected for selectivity of negatively charged phospholipids (Horvath et al, 1990). Here almost all association is through PLP and not DM-20, which implies that a functional difference may exist.

Finally it is worth noting that the observation that PLP and DM-20 encoding messages are found in cells has profound molecular biological implications. This suggests that there is not a group of PLP specific oligodendrocytes and a DM-20 specific group of oligodendrocytes within the brain. Since cells are capable of maintaining both types of messages simultaneously this shows that oligodendrocytes using the process of differential splicing of nascent messages are not programmed to make a switch one way or the other, either specifically for DM-20 or PLP encoding messages. This is particularly important as it proves that no one sequence is used to guarantee splicing out of introns in any particular message and that sequences close to the consensus sequence which has been drawn up are capable of instigating splicing.

CNP messages are found to associate almost exclusively with the cytoskeleton and generally co-localize with the protein they encode. However CNP proteins are not exclusively found with the cytoskeleton, being found in the non-cytoskeletal pool too. Generally CNP is not found in compact myelin and it has been implicated that this protein, in mature oligodendrocytes, is involved in formation of myelinating extensions (Wilson and Brophy, 1989). This role in myelinating extensions could also explain /...

explain the fact that the protein itself seems to be found in greater abundance than the messages in the peripheral areas of the cell.

The fact that CNP messages are translated on free polysomes (Gillespie et al, 1990b) also ties in with the observation that they are cytoskeletally bound.

In addition the messages encoding actin and tubulin were studied. Both messages were found to primarily co-localize with their corresponding proteins; actin in the peripheral areas of the cell and tubulin in the cell body. This observation made in oligodendrocytes is in good agreement with the work performed in fibroblasts by Lawrence and Singer (1986). Furthermore the messages encoding actin and tubulin are found in association with the cytoskeleton.

As regards cytoskeletal association of messages it is interesting to notice that translation on free polysomes does not guarantee association of messages with the cytoskeleton (e.g. MBP). However this in no way is meant to imply that translation occurs in the absence of cytoskeletal association, even though the non-cytoskeletally associated messages still retain an ability to translate in vitro. Likewise it is interesting to observe that messages which are translated on membrane-bound polysomes can also be found in association with the cytoskeleton.

However probably the most interesting observation from a molecular biological stance is the fact that oligodendrocytes have the capacity to produce two differential splicing products from the PLP gene in a single cell. Furthermore the two differentially spliced messages do not show co-localization and indeed appear to be found in completely different areas of the cell. To demonstrate any overlap of the distribution of the /...

of the two messages would require probing with two different types of reporter molecules; one radioactive and one non-radioactive.

Coupled with the work of Staugaitis et al, (1990), which suggests that there may be different roles for the MBPs, and the observation that one of the two different forms of MAG exist, depending upon the age of the animal concerned, suggests differential splicing is an integral fact of myelination. Instead of a number of different proteins performing the same function in a cell, it now seems more likely that they perform similar, but quite distinct functions within the oligodendrocyte, and hence within myelin.

At present little more can be said with any authority on the differences in function between the differential splicing products, as little is known on the overall function of each group of myelin proteins.

In summary CNP has been proposed as having a role in myelinating extensions, but is absent from compact myelin. This may suggest that the protein has some role to play in either axon finding or identification in immature cells.

The MBP gene encode 4, or more, products and the relative abundance of each varies with stage of development. Furthermore those found to increase in abundance in more mature animals have been proposed to have strong, promiscuous membrane binding properties, which may play a role in sheath compaction. Of the two proteins proposed for this role (14 and 18.5kDa) only the 14kDa MBP is necessary for apparent wild type myelination in transgenically corrected shiverer mice.

Finally the PLP protein produced from the PLP/DM-20 gene has a selective /...

a selective tendency for negatively charged phospholipids (Horvath *et al*, 1990), but DM-20 plays little part in this association - suggesting differences in the roles these two proteins play within the cell.

In summary this work has demonstrated a general co-localization of messages and their corresponding proteins, with the possible exception of DM-20. This observation with DM-20 is further compounded by the observation that a single cell has the capacity to express more than one differential splicing product at any one time. Furthermore the messages encoding these products need not co-localize, and indeed in this case they are found in completely different areas of the cell. These messages encoding the proteins investigated also show some degree of cytoskeletal association. This association can be either partial, or in some cases total. This association is found for both membrane-bound polysomes and free polysomes.

CHAPTER FIVE

FUTURE RESEARCH

5. FUTURE RESEARCH

Quite clearly this work has answered a number of interesting questions, but in so doing has generated yet more areas of potential research.

Certainly no area is more interesting than that of the cause of differential localization of the messages encoding PLP and DM-20. Since most of the message interacting with proteins, particularly those of the cytoskeleton, lies outwith the coding region (Patton and Chae, 1983; Chae and Patton, 1984),it is quite possible that there is actually a difference in the mRNA other than the absence of 105 nucleotides in the coding region. If such a non-coding sequence difference does exist, it is possible that it has some role to play in spacial segregation. By use of the probes described in Section 2.4.6. it should be possible to obtain cDNAs which are either PLP specific or DM-20 specific. These cDNAs can then be sequenced and any differences in the non-coding regions of the messages should become clear.

If however the 105 nucleotide sequence proves the sole area where a difference is detected, then this must be assumed as an area which plays an important role in spacial segregation, and that it is likely that at least part of this sequence has a role to play in interaction with proteins. Any such areas of the message which interact with proteins may be identified by nuclease digestion, as performed by Dubochet et al (1973). If the 105 nucleotide sequence, or portions therein, are protected during nuclease digestion, it would seem likely that the are involved in protein-mRNA interaction, most likely with the cytoskeleton or its associated components.

In this context /...

In this context it would appear that the PLP mRNA is held in the cell body of the oligodendrocyte. In the absence of this assumed signal (i.e. in DM-20 messages) the mRNA is free to be transported to the processes of the cell.

Aside from the differential localization of PLP and DM-20 encoding messages, a further avenue of research might be performed using animals mutant for myelin specific genes. A number of these mutants are described in Section 1.2.3., but it is worth considering their merits for biosynthesis studies. Of the myelin specific mutants already available probably the most intensely studied are shiverer and jimpy. Shiverer has already been studied at the level of in situ hybridization, and due to the size of the DNA deletion in the mutant MBP gene (around 20kb) it is a poor candidate for investigating molecular signals. However the jimpy mutant is a point mutation which results in a loss of 74 nucleotides in the mature message and so any differences detected in the localization of wild type and mutant messages might be more readily explained. This of course is assuming such differences exist.

A better route to pursue the work using myelin mutant would be use of mutants which have a mutant gene generated by some form of specifically targetted mutagenesis. Probably the best way to achieve this aim would be to synthesize genes using PCR technology, where one of the two oligonucleotides has a single mismatch nucleotide present. By selecting a number of such single base mutations it is possible that a difference in sub-cellular localization of a message may occur, or that a phenotypic difference will be observed. Such a difference will indicate an area of particular importance within the gene and so permit more detailed investigation.

The main /...

The main problem with such newly created mutants is how to study them. Obviously the ideal solution is to introduce them into the germ-line cells. Unfortunately this involves embryo injection, which although being a well established technique, is difficult to perform with a high success rate. However it is also possible that electroporation may also be a possible route for germ-line transformation.

Another approach for introducing genes into the required cells is to perform the transformation directly on cultured oligodendrocytes. This too presents problems, as oligodendrocytes are mitotically inert. Since these cells are no longer able to divide it is difficult to determine if the introduced gene has been stably incorporated into the chromosome, although use of a temperature-sensitive promoter may overcome this problem. However this does not guarantee that the introduced DNA will incorporate into the correct site in the chromosme, in order that the wild type allele already present may be nullified. Hence this would require transforming mutant oligodendrocytes with a site-directed mutant copy of the already mutant gene. The difficulties in such a method are readily obvious.

A further alternative is to use expression of the DNA in a heterologous cell system. This has already been studied in HeLa cells (Tamura et al, 1988) and fibroblasts (Barbarese et al, 1988) using MBP, but it is unclear how much information may be obtained from a system where an introduced gene is under the control of a new promoter and the protein is being expressed in an alien environment, in the absence of the normal proteins with which it may interact.

Hence in summary, use of embryo injection or electroporation is possibly /...

is possibly the best system to study newly introduced DNA. This is currently most easily studied in shiverer mice where there is a considerable portion of the native gene absent (Roach et al, 1985). Indeed transgenic mice corrected for this phenotype have already been produced (Readhead et al, 1987), to yield phenotypically wild type transgenic mice. Hence it should be a matter of repeating this work, but introducing a gene which has been mutated instead of the wild type allele.

Whichever system is used to study the effects of protein biosynthesis with mutant genes, all lines are subject to the problem that generating such large numbers of site-directed mutants is both time consuming and costly.

Hence if the DNA sequence is known, it may be possible and indeed preferential to use oligonucleotides which are complementary to specific regions of the mRNA of interest. Introducing such oligonucleotides should lead to arrest of protein synthesis. Translational arrest has already been performed by this route (Marcus-Sekura, 1988) but by targetting the non-coding regions of the message, it may be possible to extend this technique to recognition of sequences important in mRNA transportation.

While introduction of complementary oligonucleotides might not give information on the function of specific areas of the protein, it should provide information on the phenotype of a cell when the protein is located elsewhere from normal (incorrect message transportation) and also when a protein is absent (translational arrest).

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