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THE UNIVERSITY OF ALBERTA

STUDIES ON THE HUMAN MYELIN BASIC PROTEIN GENE

BY

CHRISTINE ELIANE BOUMAH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

AND RESEARCH IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (MEDICINE)

EDMONTON, ALBERTA

SPRING, 1989

ISBN 0-315-52035-4

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: CHRISTINE ELIANE BOUMAH

TITLE OF THESIS: STUDIES ON THE HUMAN MYELIN BASIC PROTEIN GENE

DEGREE FOR WHICH THESIS WAS PRESENTED: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: 1989

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A Brad et à mes

très chers parents

ABSTRACT

Myelin basic protein is suggested to be one of the antigens triggering the autoimmune attack to myelin in multiple sclerosis. DNA polymorphisms due to mutations in the coding and non-coding regions of the MBP gene are possible. The purpose of this project was first to molecular clone MBP coding sequences and then characterize MBP gene sequences in healthy individuals and MS patients.

Two c-DNA clones were purified from a c-DNA library of human brain. Size and restriction maps showed these 2 c-DNA clones, together, comprised the full length, 2.3 kb c-DNA for human MBP.

A comparison of patterns of enzyme digestion from unrelated healthy individuals revealed DNA polymorphic changes within and near the MBP gene. One group of polymorphisms was detected with restriction enzymes EcoRI, BamHl, BglII, HindIII, KpnI, PstI and PvuII. The mutation involved insertions-deletions of 300 to 400 bp which mapped around exon 3 of the gene as shown by hybridization to an exon 3-specific oligonucleotide probe. A second group of DNA polymorphisms mapped in the 5' flanking region of the gene; this was also an insertion-deletion mutation, detected with the restriction enzymes PstI, PvuII, HincII and SalI. The latter polymorphism, assessed by the presence of 3.0 kb PvuII and 1.0 kb HincII alleles, occurred with a frequency of 28.1% among unrelated healthy individuals.

The frequency of 3.0 kb PvuII - 1.0 kb HincII alleles was 35.3% among 34 MS patients. This indicated no direct linkage

between susceptibility to MS and PvuII-HincII polymorphism in the 5' flanking region of MBP gene. The 3.0 kb PvuII and 1.0 kb HincII alleles occurred as frequently in females with MS as in healthy females. The frequencies of PvuII-HincII polymorphism measured for patients with progressive MS, for patients with relapsing-remitting MS, for patients who developed MS before age 25 and for patients who developed MS between 25 and 40 were 35.3%, 25.0%, 35.7% and 29.4% respectively.

ACKNOWLEDGEMENTS

I wish to offer my thanks and appreciation to my supervisor, Dr. Wai-Choi Leung for his support and willingness to share his thoughts, ideas and in the preparation of this manuscript. I would also like to show my appreciation to my co-supervisor, Dr. Lorne Tyrrell for his kindness, daily encouragement and for providing proper working environment.

I acknowledge the role of Dr. Bill McBlain who devoted so much time and efforts to carry me over several obstacles to the completion of these studies. I am greatly indebted to Dr. Ross Hodgetts for the time spent scrutinizing my data and for his constructive criticism. I am also very grateful to Dr. J. Preiksaitis and the late Dr. J. G. Kaplan for their direction and interest in my progress. By providing blood samples from the MS clinic, Dr. K. Warren has made this research possible, I sincerely thank him. A special thanks goes to Dr. Sheila Schmutz from Saskatoon, Saskatchewan, for graciously consenting to be my external examiner. Ms Heather Mitchell who has expertly typed this manuscript, Richard Sherburne and San Vinh from Medical Microbiology "Photos" deserve my sincere thanks for their work and also for their patience and understanding in the face of rush deadlines.

Une pensée à la mémoire de mes chers disparus: Tata, Tébébé, Nganga, Grand-Papa, Tonton Ngoleine et ce cher Dr. Kaplan.

This work was supported by the Alberta Heritage Foundation for Medical Research in the form of a studentship and a research allowance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

apo-	apolipoprotein
CF	cystic fibrosis
c-Ha-ras	cellular Harvey sarcoma protooncogene
CM	centimorgan
CNPase	2',3'-cyclic nucleotide-3'-phosphohydrolase
CNS	central nervous system
CSF	cerebrospinal fluid
DMT	dimethoxytrityl
EAE	experimental allergic encephalomyelitis
HDL	high density lipoprotein
IgG	immunoglobulin G
LDL	low density lipoprotein
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MHC	major histocompatibility system
MS	multiple sclerosis
MSNT	mesitylene sulfonyl nitrotriazolide
Pfu	plaque-forming unit
PLP	proteolipid protein
PNS	peripheral nervous system
RFLP	restriction fragment length polymorphism

CHAPTER 1

LITERATURE REVIEW

I. Structure and Composition of Myelin in the Central Nervous System

Myelin is the multilayered membrane that surrounds nerve fibers of both the peripheral and the central nervous systems (CNS). It acts as an electrical insulator to facilitate the transmission of nerve signals. In an unmyelinated fiber, local continuous depolarizations all along the nerves are necessary for propagating the nerve impulse. The myelinated axon can only be excited at the nodes of Ranvier, which are small gaps of exposed nerve membrane. The nerve impulse is therefore transmitted from node to node, in a saltatory and more rapid manner.

The CNS myelin is synthesized and assembled by oligodendrocytes, which are glial cells scattered among nerve cells. When the axon has reached the proper size and the glial cell has received some signal, possibly from the axon , myelination starts (Poduslo *et al.*, 1984). The oligodendrocyte extends a process around the nerve fiber, extruding the cytoplasm and fusing together the inner cytoplasmic faces of the membrane extension. The membrane process then wraps itself successively around the nerve, in a concentric manner. After compaction of the membrane layers, the characteristic multilamellar structure of myelin is generated. When observed under electron microscopy, myelin appears as a series of alternating electron dark and light

lines. The dark or electron dense lines result from the fusion of the inner cytoplasmic faces of the glial cell membrane while the electron light bands are created by the stacking of the outer faces of the membranes.

Although derived from a plasma membrane, myelin differs from normal membrane in its molecular composition. One of the most unique features of myelin is the unusually high lipid to protein ratio (about 75:25) while in plasma membranes, lipid and protein exist in nearly equal amounts, for example 55:45 for the oligodendrocyte membrane (Poduslo, 1975). Another special feature of myelin membrane is its protein composition: there is less protein and also fewer types of proteins. Two major proteins predominate, the myelin basic protein (MBP) which represents 33% of total protein and the proteolipid protein (PLP), or lipophilin, representing up to 50% of myelin proteins. The other proteins are the 2',3'-cyclic nucleotide-3'-phosphohydrolase (CNPase) (5% of total protein) and the myelin-associated glycoprotein (MAG) (less than 1% of myelin proteins).

How myelin components and which component(s) of myelin are involved in organizing and/or maintaining the multilamellar structure has been the subject for intensive investigation.

A. Lipids

X-ray diffraction data on native myelin show lipids organized in bilayers, an arrangement that is typical of membrane lipids where polar lipid head groups are bounding a

central hydrocarbon core. When the total lipids were isolated from myelin and reconstituted in the absence of myelin proteins, they still formed bilayers. The ability to organize bilayers is therefore a property that could be assigned to lipids in myelin. It is, however, not specific for myelin lipids and could not account for the unique structure of myelin. Furthermore, there is no lipid class specific to myelin. Cholesterol, phospholipids and glycolipids are present in myelin, in molar ratios (4:3.5:2) similar to those reported for other plasma membranes (Norton and Cammer, 1984). Galactocerebroside, which is a relatively rare glycolipid, is found in high amounts (approximately 20% of myelin dry weight) in myelin but again is not a myelin-specific lipid. Myelin also contains 6% phosphatidylcholine, as opposed to 30% in plasma membranes. Multilamellar membranes with structures similar to myelin's, such as the grana thylakoid membranes or the rod outer segment disk membranes contain no galactocerebroside (Braun, 1984) and have phosphatidylcholine levels near 30% (Kirschner *et al.*, 1984). With respect to lipids, the only unique feature in myelin is the high lipid proportion. However, membrane stacking occurs in grana thylakoid membranes and rod outer segment disk membranes which have lipid protein ratios of 60:40 (Li, 1978) and 50:50 (Daemen, 1973), respectively.

From these considerations, it appears that the multilamellar arrangement with membrane stacking observed in myelin and other systems depends on neither a particular

lipid class nor does it depend on a high lipid content. The presence of large amounts of lipids is, however, not fortuitous and must contribute to myelin's function as an insulator. Lipids effectively exclude water and water-soluble compounds including the sodium ions and potassium ions which are essential in membrane depolarization.

B. Proteins

The myelin protein repertoire is very small compared to that of other biological membranes; only a small number of polypeptides is present, most of which, like MBP and PLP, are found only in myelin and myelin-forming cells. PLP is even restricted to CNS myelin since Schwann cells rarely express PLP (Puckett *et al.*, 1987). In the peripheral nervous system (PNS), PLP is replaced by protein Po (Lemke and Axel, 1985). It is likely that the unique features of myelin are derived from the protein fraction and that both PLP and MBP, which together comprise 80% of the total proteins, play essential structural roles.

1. Proteolipid Protein

The main characteristic of PLP is its solubility in organic solvents, a lipid-like feature from which the name proteolipid was derived. PLP is composed of 2 types of polypeptides, the DM-20 and the actual PLP (Macklin *et al.*, 1984). DM-20 is still poorly characterized but it appears to have a molecular weight approximately 5 kDa lower than PLP. DM-20 and PLP have common amino- and carboxyl-terminal amino

acids (Vacher-Lepretre, 1976) and are immunologically related (Trifilieff *et al.*, 1986). Their different mobilities in SDS-PAGE gels may result from factors such as charge microheterogeneity, posttranslational modifications, conformational differences or differential SDS binding (Lees *et al.*, 1981). Alternatively, DM-20 may be identical to PLP but contain some internal sequence deletion as suggested by Trifilieff and coworkers (1987).

The primary amino acid sequence of PLP has been determined; it has a molecular weight of 30 kDa and, as expected for proteins with important functions, it is highly conserved. There is a complete homology (based on both protein and c-DNA sequences) between the human and the rat proteins (Milner *et al.*, 1985) and a 94% homology with the bovine protein (Stoffel *et al.*, 1985). PLP is exceptionally hydrophobic with its 276 amino acid residues arranged into 5 long and strongly hydrophobic segments that are linked by 4 short hydrophilic, highly charged sequences. There are 14 cysteine residues but only 4 residues will form thiol groups with sulfhydryl reagents (Lees *et al.*, 1969). The remaining cysteine residues account for the 5 disulfide bonds suggested to bridge the distant hydrophilic sequences of the molecule (Cockle *et al.*, 1980). Another feature of the chemical composition of PLP is the presence of fatty acids (2-4% by weight) to approximately 3 moles per mole protein. Fatty acid residues are usually attached by an ester bond to a threonine residue within one of the hydrophilic segments

(Stoffel *et al.*, 1983). The biological role of esterified fatty acids in the myelin PLP remains unknown but covalently bound fatty acids are believed to increase the hydrophobicity of proteins (Lees and Brostoff, 1984).

Because of the high hydrophobicity, PLP is likely to be an intrinsic membrane protein that could insert itself deeply within the lipid bilayer, in the protein-rich hydrocarbon milieu. This assumption is supported by indirect evidence from studies utilizing x-ray diffraction, chemical probes and bifunctional crosslinking reagents. In electron micrographs, PLP can be located on the electron light bands of myelin, extending in the lipid layers (Braun, 1977). Since the myelin light bands are the bands formed when the external faces of the glial cell membrane are brought in contact, Boggs and Moscarello (1978) postulated that PLP must cause adhesion of the bilayers through hydrophobic interactions with adjacent lamellae. Two models have been proposed for the molecular arrangement of PLP in myelin, explaining how it could mediate interactions between opposed membranes. In both models, 3 of the 5 hydrophobic segments of PLP are transmembrane domains, long enough to span the 47 Å bilayer; the other 2 hydrophobic segments are not as deeply embedded in the membrane. In Laursen's model, interactions occur between the 2 non-transmembrane hydrophobic segments in adjacent membranes (Laursen *et al.*, 1984), while in Stoffel's model, the surface hydrophobic segments together with some of

the short hydrophilic sequences intera toffel *et al.*, 1984).

2. Myelin basic proteins

The second most abundant class of myelin proteins is the myelin basic proteins. It consists of a group of 4-5 structurally related polypeptides, ranging in size from 21.5 kDa to 14 kDa with an 18.5 kDa polypeptide as the predominant form in adult human CNS myelins. MBPs from human, cow, sheep, rabbit, rat and mouse have been sequenced; the proteins are very highly conserved showing sequence homologies (between the above species) greater than 85%. The amino acid sequence analysis reveals very basic proteins with a high content of arginine (18 for 170 amino acids), lysine (13 residues for 170 amino acids) and histidine (10 residues total). Most of the acidic residues, glutamic acid and aspartic acid, are amidated. The overall charge of MBP is strongly positive with an isoelectric point greater than 10.

Because of its basic nature, MBP interacts with acidic lipids. When mixed with myelin lipids, ordered multilamellar structures are formed which have repeat periods (154 Å) similar to those of native myelin (Mateu *et al.*, 1973; Kirschner and Caspar, 1977). Other basic proteins such as cytochrome C, lysozyme and poly-L-lysine, mixed with total myelin lipids will also form ordered lamellar structures, but these have repeat periods of only 80 Å. The fact that MBP interacts differently with the acidic lipids when compared to

the other basic proteins suggests that charge neutralization alone, although important, does not account for the periodic structure of myelin.

MBP is an extrinsic membrane protein shown by electron microscopy to associate with the cytoplasmic, inner faces of myelin, both in the lipid head groups and in the aqueous phase. At this location, one can reasonably assume that MBP is the element involved in the tight compaction of the inner membrane faces to yield the myelin dark lines, an essential role in the stabilization of the multilayer organization. One mechanism proposed to explain the function of MBP suggests that MBP molecules interact with phospholipids on adjacent lamellae thereby acting as a proteinaceous "glue" to bind the lamellae together (Boggs *et al.*, 1982). Support for this mechanism has been given by the isolation of MBP molecules with covalently attached phosphotidylinositol (Yang *et al.*, 1986). Another mechanism suggests that the adjacent membrane lamellae are bound together through the interaction of MBP molecules located on opposite surfaces (Carnegie and Moore, 1980). MBP has recently been shown to self-associate in interactions that are not electrostatic in nature (since they are ionic strength and temperature-dependent), in contrast to the interactions between MBP and cytochrome C or lysozyme which have an electrostatic component (Moskaitis *et al.*, 1987). The interaction between individual MBP monomers is probably not very strong (Burns *et al.*, 1981). However, the concentration of MBP at the major electron-dense line has

been estimated to be so high that the MBP self-association could possibly be a physiologically meaningful force in maintaining myelin organization (Lampe *et al.*, 1983).

3. The 2',3'-cyclic Nucleotide-3'-phosphohydrolase

CNPase was the first enzyme to be unequivocally characterized as a component of myelin (reviewed by Vogel and Thompson, 1988). Immunological and structural studies showed CNPase was in fact identical to the high molecular weight Wolfgram myelin proteins (Wolfgram and Kotorii, 1968), the 2 Wolfgram doublets with molecular weights 44 kDa and 47 kDa corresponding to the 2 subunits of CNPase (Drummond and Dean, 1980).

The enzyme CNPase is unique among mammalian tissues in converting 2',3'-cyclic nucleotides to 2'-phosphate products. Since 2',3'-cyclic nucleotides are not known to occur in mammalian brain, the role and even the true substrate for CNPase in CNS remains obscure. The role of CNPase is not known either in the retina where it is found in very high concentrations in the inner segments of photoreceptor cell membranes (Giulian *et al.*, 1983). Recently, it has been proposed that CNPase may function as an RNA ligase (Tippins and Sprinkle, 1987). Eukaryotic RNA ligases, particularly tRNA ligases, have 3 associated enzymatic activities, the CNPase, the 5' polynucleotide kinase and the RNA ligase, all of which reside in a single polypeptide chain (Greer *et al.*, 1983). CNPase has been shown to phosphorylate the 5'-hydroxyl groups of tRNA (Sprinkle *et al.*, 1987) and both

CNPase subunits can be adenylated *in vitro*, representing an intermediate step in the RNA ligation reaction (Sprinkle and Tippins, 1987). There is however, little RNA present in myelin (Autilio *et al.*, 1964). Furthermore, CNPase enzymes exist that have no associated RNA ligase (Tyc *et al.*, 1987). The observation that CNPase levels increase rapidly during myelination (Cammer and Zimmerman, 1981; Reynolds *et al.*, 1987) does suggest a role for these enzymes in the myelination process. However, because they exist at a much lower percentage as compared to MBP and PLP, CNPases are probably more important functionally than structurally in the myelin sheath.

4. Myelin-associated glycoprotein

MAG is a relatively minor component of CNS myelin, comprising less than 1% of the proteins. MAG has a molecular weight of approximately 100 kDa, of which about 30% is carbohydrate (Quarles *et al.*, 1983). Originally, MAG was thought to be confined to surface membranes of myelin-forming cells, in the periaxonal and non-compacted areas of the myelin sheath (Trapp and Quarles, 1982). However, immunocytochemistry studies by Webster and coworkers (1983) revealed MAG presence in compact myelin as well and these authors suggested the name myelin glycoprotein, MGP, rather than MAG since this glycoprotein is an integral myelin component.

The function proposed for MAG is in the axonal-oligodendrocyte interaction where it maintains the periaxonal

space by preventing the "complete" compaction of myelin-forming cell and myelin membrane. Some authors have suggested a role for MAG as the signal for myelination during development (Morell and Norton, 1980). Two types of MAG, also called 1B236 in rat brain, have been isolated; they differ slightly in their structure and are developmentally regulated (Frail and Braun, 1984). The immature rat brain has a MAG with a higher molecular weight than the MAG in adult rat brain (Quarles *et al.*, 1973). This higher molecular weight-MAG, has been also isolated from the developing brain of other animals (Matthieu *et al.*, 1974). The 2 rat MAG, p72 MAG and p67 MAG have 626 amino acid residues and 582 amino acid residues, respectively. They share a single membrane-spanning domain and a glycosylated amino-terminus but differ in the structures of their carboxy-termini. The mRNAs encoding p72 MAG and p67 MAG have been fully characterized. The p72 MAG mRNAs appear during rapid myelination, in the early developmental stage while p67 MAG mRNAs become dominant only when the rate of myelination decreases (Frail *et al.*, 1985). These mRNAs are 2 distinct molecules which have recently been shown to be derived from the alternative splicing of a common primary transcript (Lai *et al.*, 1987; Salzer *et al.*, 1987).

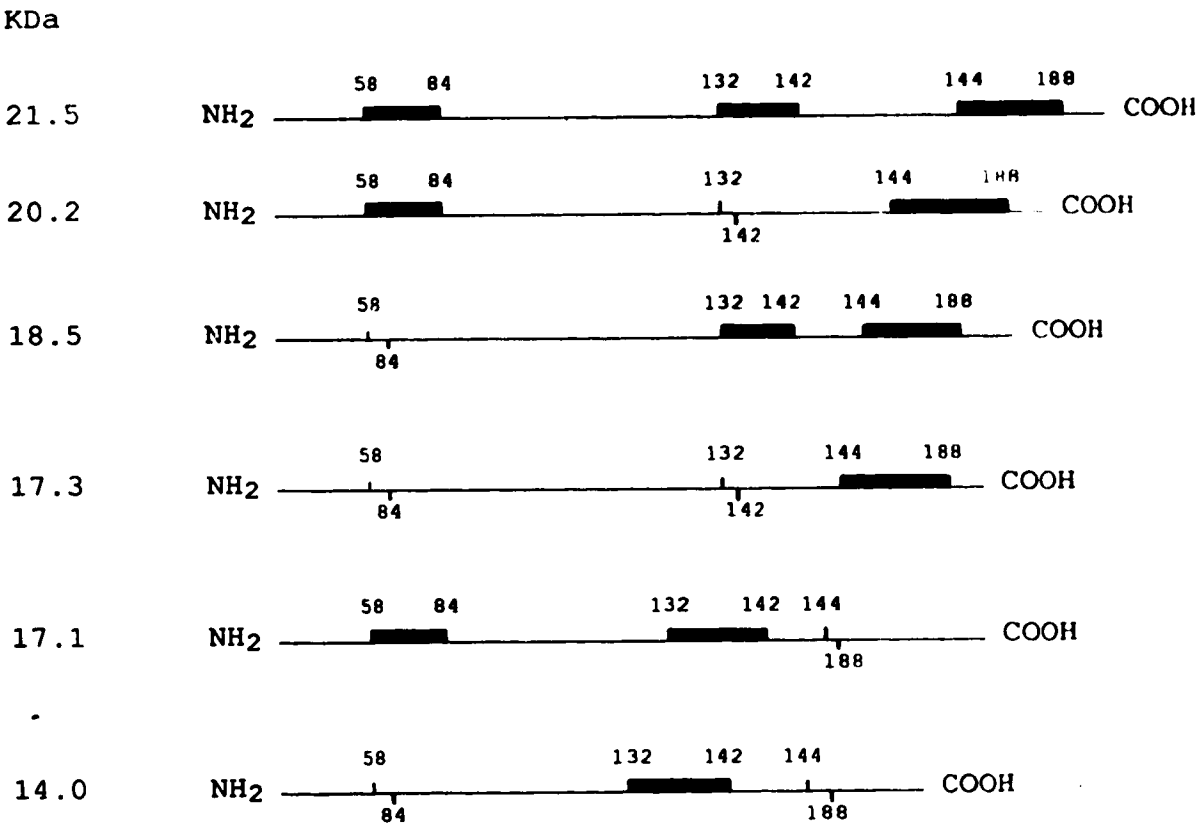
In mice, the quaking mutation is characterized by a severe deficiency in myelin with lack of compaction of myelin lamellae in both the CNS and the peripheral nervous system. It is due to premature cessation of the differentiation of

myelin-forming cells with a failure of myelin assembly (Hogan and Greenfield, 1984). The MAG isolated from quaking mice is the high molecular weights, similar to the p72 MAG of the immature rat brain (Inuzuka *et al.*, 1985). These reports led Baba and coworkers (1987) to the conclusion that decreasing high molecular weight-MAG must be a prerequisite for normal compaction and maturation of myelin.

II. The Different Myelin Basic Protein Molecules

Five different MBPs a c nd in mouse and rat brains with molecular weights of 2. .5 .a, 18.5 kDa, 17.3 kDa, 17.1 kDa and 14 kDa. The human CNS myelin contains 4 different basic proteins; three, the 21.5 kDa, the 18.5 kDa and the 17.3 kDa are homologous to the mouse proteins while the fourth MBP with molecular weight 20.2 kDa is specific for humans (Roth *et al.*, 1986). There have been reports on the presence of some 20 kDa basic proteins in mice (Sorg *et al.*, 1986), rats (Agrawal *et al.*, 1986), rabbits (Gilbert *et al.*, 1982) and ox (Waehneldt *et al.*, 1985) but their relationship to the known MBPs has not been clear until now. There is no human MBP equivalent to the rodent 14 kDa protein. All the MBPs are structurally related and have common amino-terminal and carboxy-terminal sequences (Fig. 1). The human 20.2 kDa is identical to the larger 21.5 kDa except for the deletion of 11 amino acid residues between amino acids 132 and 142 (Roth *et al.*, 1987). Similarly the 18.5 kDa MBP is

Fig. 1. Schematic representation of the different forms of MBP. Thick lines represent peptide sequences encoded by exons 2, 5 and 6 which can be deleted to give rise to smaller MBP variants. The deleted sequences, amino acids 58 to 84, 132 to 142 and 144 to 188, correspond to those encoded by exons 2, 5 and 6, respectively, of the MBP gene. The 20.2 KDa MBP form is human-specific; the 14.0 KDa MBP is absent in humans.


KDa
21.5
NH2
58
84
132
142
144
188
COOH
20.2
NH2
58
84
132
142
144
188
COOH
18.5
NH2
58
84
132
142
144
188
COOH
17.3
NH2
58
84
132
142
144
188
COOH
17.1
NH2
58
84
132
142
144
188
COOH
14.0
NH2
58
84
132
142
144
188
COOH

homologous to the 21.5 kDa but is missing a 26 amino acid-peptide near the amino-terminus. The 17 kDa MBPs have only recently been resolved in mice as 2 distinct proteins (Newman *et al.*, 1987): the more abundant 17.1 kDa is missing amino acids 144-188 of the large MBP; the minor 17.3 kDa MBP is missing both the 26 amino acid-peptide in the amino-terminus (amino acids 59-84) and the 11 residues between amino acids 132 and 142. Finally, the 14 kDa protein variant is also identical to the 21.5 kDa but is missing the 26 amino acids residues in the amino-terminus as well as the 44 residues, amino acids 144 to 188, in the carboxyl-terminus.

The reason for so many different MBP molecules is not clear and is still, to a large extent a matter of speculation. The effects of deletions of peptide fragments on the structure and function of the MBP variants is not well understood either. All these MBP variants still have high isoelectric points, ranging from 12.2 to 12.4. Although MBP is largely hydrophilic in nature, it does contain 5 small hydrophobic regions (Weise, 1985). Spectroscopic methods indicate the presence of a significant amount of beta-structure in MBP (Randall and Zand, 1985), and several theoretical models of the 18.5 kDa MBP structure have been proposed. In these models, the 5 hydrophobic regions are arranged into a five-stranded beta-sheet structure (Stoner, 1984; Smith, 1985; Martenson, 1986). One of these 5 strands, amino acids 135-143, comprises the deleted peptide

in the 20.2 kDa and the 17.3 kDa MBPs and the deletion of amino acid residues 144 to 188 observed in the 17.1 kDa and the 14 kDa proteins include another beta-strand. The structure proposed for MBP would, therefore, be significantly altered in the 20.2, 17 and 14 kDa MBPs and this should be reflected in the function of the protein.

The relative concentration of these MBP variants changes during aging; in general, there is a gradual decrease in the high molecular weight species and an increase in smaller MBPs in older brains (Chanderkar *et al.*, 1986). For example, the 14 kDa and the 18.5 kDa MBPs are the most abundant MBPs in adult mice brains; the 21.5 MBP is present but in very low concentrations, the ratio for the 21.5 kDa:18.5 kDa:14 kDa polypeptides being 1:10:35 in adult mice (Barbarese *et al.*, 1978). Similarly, the human 20.2 kDa is expressed early during myelinogenesis in the fetal brain; its expression gradually decreases with development until the adult stage where it is completely absent; concomitant with this, is an increase in the levels of the 18.5 kDa and 17 kDa human protein variants. It seems that different forms of MBP have particular functions during myelination, the large MBPs may have significant roles during the initial phases of myelin deposition whereas the smaller MBPs may be essential in the maturation process or possibly in the repair of the myelin membrane in case of damage.

Within the adult brain, there are local variations in the structure of myelin and the CNS can be separated into myelin fractions of different densities. Some parts of the brain contain a light myelin which is enriched in compact multilamellar structures while other parts of the brain are made of denser myelin containing fewer lamellae and loose myelin membrane fragments (Waehneldt, 1978). These denser myelin fractions have been suggested to reflect less stable myelin structures derived from newly formed or immature myelin (Pereyra and Braun, 1983). While lipids, MAG and the Wolfgrams content were similar between the myelin fractions, Cruz and Moscarello (1985) found the dense myelin in human brain contains mainly large MBPs with very small amounts ,if any, of the lower molecular weight MBPs.

In addition to the multiple MBP variants with different molecular weights, charge microheterogeneity has been observed. In neutral SDS gels, a single MBP variant migrates as a single band, however, at alkaline pH, this single MBP can be separated into several charge isomers by electrophoresis or cation exchange chromatography. This charge microheterogeneity has been ascribed to posttranslational modifications such as phosphorylation (Martensen *et al.*, 1983), deamidation (Chou *et al.*, 1976) and in some cases, C-terminal arginine loss (Chou *et al.*, 1977).

MBP is phosphorylated both *in vitro* and *in vivo* (Turner *et al.*, 1982) by different protein kinases (Ca^{2+}-independent, phospholipid and Ca^{2+}-dependent or protein kinase C, Ca^{2+}-calmodulin-dependent and cyclic-AMP-dependent). Phosphorylation of many serine and threonine residues occurs but which sites are phosphorylated *in vivo* has not been established. The phosphorylation of even a single site will decrease the overall MBP charge.

Most of the glutamic acid and aspartic acid residues in MBP are amidated, deamidation will also render the molecule less basic. Similarly, removal of the two carboxy-terminal arginine residues of MBP, if it occurred *in vivo* , will decrease MBP charge. Any change that decreases the positive charge on MBP has been found to affect MBP function. Brady and coworkers (1985) have shown by utilizing *in vitro* phosphorylated MBP, that a difference of one charge has a very pronounced effect on the ability of MBP to organize lipid bilayers into crystalline multilayered structures similar to intact myelin. The more cationic of the MBP charge isomers was more effective at inducing the formation of multilayers. Cheifeitz and Moscarello (1985) have also found that removal of the two carboxy-terminal arginine residues *in vitro* greatly decreased the ability of the MBP isomer to induce lipid aggregation. Looking at the distribution of the charge isomers in different myelin fractions of the brain, Cruz and Moscarello (1985) found that

the light myelin fractions with compact myelin, were enriched in the more positively charged basic proteins whereas these components were reduced significantly in the denser myelin fractions. Similarly, different charge microheterogeneity was found for human myelin basic proteins isolated from myelins of a 2-year-old and an adult brain. The MBP of the 2-year-old had fewer components, mainly the most cationic isomers and the less positively charged MBP isomers (associated with less dense, stable myelin fractions) were absent.

Another modification of MBP with possible effect on function is methylation. MBP is methylated specifically at arginine residue 108 which exists as a mixture of unmethylated (8%), monomethylated (61%) and dimethylated (31%) forms (Young and Grynspan, 1987). The methylation is catalyzed by a S-adenosylmethionine arginine methyl transferase present in myelin. It has been postulated that the introduction of a methyl group to the side chain of an amino acid induces the hydrophobicity of the protein (Brostoff and Eylar, 1971) thus influencing the interaction of MBP and phospholipid molecules in the formation of the myelin sheath. Using an inhibitor of protein methyltransferases, sinefungin (an analogue of S-adenosylmethionine), Amur and coworkers (1986) observed that inhibition of the methylation of MBP causes the inhibition of compact lamellar formation in myelin-like processes produced

by cultured cerebral cells from embryonic mice. Chanderkar and coworkers (1986) have studied MBP methylation during mouse brain development. They reported higher ratios of MBP methylation to synthesis or methylation index (methyl-[^{3}H]-methionine/[^{35}S]-methionine) in younger brains. This correlated with increased MBP-specific methyltransferase activity and a higher S-adenosylmethionine pool (Gharib *et al.*, 1985) indicating that methylation is most active in MBP during the period of MBP synthesis and myelination. MBP from carp is the only MBP studied so far that does not contain methylarginine residues (Deibler *et al.*, 1973). When comparing abilities to induce aggregation of unilamellar vesicles prepared from myelin suspensions, the methylated MBP from cows and other species was more efficient than the nonmethylated carp protein which was no more effective than lysozyme or polyhistidine (Young *et al.*, 1987). Methylation of arginine residue 108 in MBP is, therefore, important for the interaction with the myelin membrane.

In summary, each MBP variant has a function at some stage during development and/or in some parts of the brain. High molecular weight-MBP variants and highly methylated MBPs are needed to organize myelin in immature brains. The compact myelin found in most parts of adult brain consists of lower molecular weight forms of MBP. These variants have less beta-stranded structures, are highly basic and are poorly methylated. In the dense, less stable myelin

fractions in brains of all developmental stages, the high molecular weight, less positively charged and highly methylated MBPs are found.

III. Organization of MBP Gene in Animals

In vitro translation experiments have established that the different MBP size variants are encoded by different mRNAs (Yu and Campagnoni, 1982; Carson *et al.*, 1983). The first MBP c-DNA was isolated using synthetic DNA probes derived from the reverse translation of the amino acid sequence of the rat protein and coded for the abundant, small 14 kDa rat MBP (Roach *et al.*, 1983). Thereafter, the c-DNAs coding for the other rat (Campagnoni, 1985) and mouse MBPs (Zeller *et al.*, 1984) became available. These c-DNAs with a size of approximately 2.2 kilobases(Kb) are very long, considering that no more than 600 bases are needed to specify the large 21.5 kDa MBP. As expected from protein sequences, all these mRNAs show similar organization, with the mRNA for the 21.5 kDa MBP containing all the sequences absent from the other mRNAs. All the MBP mRNAs in mice and rats have a short, 47 base 5' untranslated region followed by a relatively short coding sequence (585 bases in the large MBP) and a long, 1488 base 3' untranslated region containing polyadenylation signals. There are 2 possible open reading frames with splice acceptor sites near their beginning in the 3' untranslated region.

Mouse MBP genomic sequences were obtained from a cosmid library. Since Southern blot analyses of these genomic sequences reveal a simple pattern, similar to the one predicted by the restriction maps, it was concluded that mice and rats contain a single MBP gene (Takahashi *et al.*, 1985). The MBP gene has been mapped to the distal end of mouse chromosome 18 (Barnett *et al.*, 1985). The mouse MBP gene is 32 Kb long with 7 relatively small exons, interrupted by very long introns. Exon 1 encodes the first 56 amino acids of mouse MBP, which differ from the rat sequence at 3 positions. Exon 2 encodes amino acids 58 to 84, differing from the rat sequence at a single site. The third exon is only 36 base pair long and encodes amino acids 91 to 102, none of which differs from the rat sequence. Exon 4, 33 base pair long encodes amino acids 103 to 113, with no changes from the rat. Exon 5 specifies amino acids 132 to 142. Exon 6, encoding amino acids 144 to 188, and exon 7 have not been fully sequenced yet. The 21.5 kDa MBP contains information specified by all the 7 exons while the peptide encoded by exon 2 is missing in the 18.5 kDa MBP, in one of the 17 kDa MBP (the 17.3 KDa), which is also lacking information from exon 5, and in the small 14 kDa protein. The small MBP as well as the second 17 kDa MBP (17.1 kDa) are both missing the peptide specified by exon 6 sequences (Fig. 1). Therefore, all the different mouse and rat MBP mRNAs are generated from a single gene through alternative splicing of exons 2, 5 and

6 (de Ferra *et al.*, 1985). No frameshifts are produced by these alternative splicings, the 6 exon-exon unions in the 21.5 kDa MBP mRNA falling precisely between codons.

IV. Structure of the Human MBP Gene

The c-DNAs coding for all the 4 human MBP species have been molecular cloned and their sequences compared. As in animals, the human MBP mRNAs differ from each other by deletion(s) or insertion(s) of internal sequences. The human mRNAs are also approximately 2.2 Kb in size, have short 5' untranslated regions of 36 base pairs, coding sequences of about 500 base pairs and a long, 1603 base pair 3' untranslated region with a polyadenylation signal. The 3' untranslated portion here contains only one possible open reading frame which, unlike the rodents, does not have a splice acceptor site (Kamholz *et al.*, 1986).

Southern blotting studies of human genomic DNA reveal simple patterns, consistent with a single human MBP gene (Kamholz *et al.*, 1986). The MBP gene maps on the tip of the long arm of human chromosome 18, region 18q22 to 18qter (Saxe *et al.*, 1985; Sparkes *et al.*, 1987). The 21.5 kDa protein is also the largest human MBP species, with the 18.5 kDa protein as major adult MBP; the sequences deleted from the 21.5 kDa MBP to generate the other human MBPs correspond exactly to exons 2 and 5 of the mouse MBP gene (Fig. 1).

Mouse and human exons 2 specify peptides identical in 22 of 26 amino acids. This suggests that human and mouse genes have similar exon structures and that human MBP mRNAs are also generated by alternative splicing of a primary MBP transcript. No mature human MBP mRNA missing exon 6 sequences (i.e. equivalent to the mouse 17.1 kDa and 14 kDa MBP) has been identified. Unlike the mouse the 4 human MBP mRNAs must arise by splicing of only exons 2 and 5 from the primary gene transcript. This indicates that the predominant MBP splicing pathways in human and mouse might be different.

Alternative splicing is a common and efficient mechanism to generate protein diversity; this could happen in a number of ways. For example, different transcripts can be generated from a single primary transcript through the use of different promoters such as seen for the mouse α-amylase gene (Schibler *et al.*, 1983). In this case, the transcripts differ at their 5' termini. Related transcripts that are different at the 3' ends and are spliced differently are generated when 2 different polyadenylation signals are used as seen for the calcitonin gene (Amara *et al.*, 1984). Finally, primary transcripts with identical termini are alternatively spliced to yield different mature mRNAs; this has been observed in the genes for fibrinogen (Crabtree and Kent, 1982), lens α-A crystallin (Piatigorsky, 1984) and Troponin T (Breitbart *et al.*, 1985). Human and mouse MBP genes belong to this last group. The other myelin proteins like PLP, MAG and CNPase

also exist in more than one form. The 2 forms of PLP have been suggested by Trifilieff and coworkers (1987) to be encoded by 2 different mRNAs having identical 5' and 3' termini; similarly Lai and coworkers (1987) proposed alternative splicing as the mechanism by which the 2 MAG mRNAs, different at their 3'-termini are generated and, although only one mRNA species has been isolated so far (Kurihara *et al.*, 1987; Vogel and Thompson, 1987), alternative splicing could also be invoked to justify the existence of 2 CNPase components (Vogel and Thompson, 1988). It is possible that alternative splicing is an important, frequently employed mechanism in the oligodendrocyte.

V. MBP in Myelin Disorders

A. Demyelinating diseases of animals

The function proposed for MBP is to maintain the cytoplasmic faces of the myelin membrane in close apposition, in a compact enough manner so as to generate and maintain the "major dense line" of myelin. It is conceivable that defects or absence of MBP result in changes in the structure or even loss of myelin, with adverse consequences on neurological function(s). Severe injury and death of neurons cause demyelination. This is referred to as secondary demyelination as opposed to primary demyelinations where a damage is done directly to myelin or to oligodendrocytes.

The demyelinations associated with defects in myelin proteins, for example, PLP in Jimpy disease (Hudson *et al.*, 1987; Ikenaka *et al.*, 1988), MAG in quaking mutation (Davisson *et al.*, 1986; Fujita *et al.*, 1988) and MBP in shiverer disease (Kimura *et al.*, 1985), where the nerve axons remain relatively intact, belong to the latter category. The shiverer disease of mice is one of the best characterized of myelin disorders; it is an autosomal recessive mutation affecting young mice at time of onset of CNS myelination. The shiverer mice appear normal for the first 12-14 days after birth then start shivering, later have convulsions of increasing strength until they die by 4 months. Normal mice live 24-36 months. The brains of shiverer animals contain very little myelin. There is no evidence under electron microscopy, of myelin major dense line (Rosenbluth, 1980) but myelin sheaths in PNS appear normal (Peterson and Bray, 1984). The only known defect in these mice is the absence or a low level of MBP (Jacque *et al.*, 1983). Very little mRNA for MBP is present and the MBP gene in shiverer mice has been shown to contain a large deletion extending from exon 2 through the 3' end of the last exon (Molineaux *et al.*, 1986). Furthermore, the shiverer mutation has been mapped to chromosome 18, at the same location as the mouse MBP gene (Sidman *et al.*, 1985). This suggests that MBP gene deletion might be responsible for the shiverer phenotype.

Confirmation for the role of MBP defects in the shiverer disease has been given in the recent gene therapy experiments by Readhead and coworkers (1987) and Popko and coworkers (1987). They have introduced copies of MBP gene into the germ line of homozygous shiverer mice to obtain transgenic shiverer mice expressing one or 2 copies of the MBP transgene. The shiverer mice with 2 copies of MBP gene were producing compact myelin and most of their axons in the optic nerve were properly myelinated; the shiverer transgenic mice with a single copy of MBP gene also made some myelin, but only the larger axons were myelinated. These mice still had some convulsions, although at lower frequency than in the homozygous non-transgenic shiverer mice and they lived slightly longer. The transgenic mice which expressed 2 MBP copies showed near normal phenotypes: they only shivered occasionally, had no convulsions and lived a normal life span. With 2 copies of MBP gene, transgenic shiverer mice did make approximately twice the amount of MBP found in the mice containing only one MBP copy. Besides proving that defects in MBP gene are sufficient to cause severe neurological disorders, these data show a clear relationship between the level of MBP gene expression, degree of myelination of axons in the CNS and severity of disease.

Another MBP-associated disorder in animals is the "myelin-deficiency" of mice which is allelic to the shiverer mutation, is also recessive (Hogan and Greenfield, 1984) and

displays a very similar but less severe phenotype (longer life than shiverer mice) (Shen *et al.*, 1985). "Myelin-deficiency" is not due to a deletion in the MBP gene but to duplication of some parts of the gene (including the 5'-regulatory portion) which affects gene transcription (Okano *et al.*, 1987; Akowitz *et al.*, 1987). The shiverer mutation is dominant over "myelin-deficiency" as shown by CNS myelin structure and disease pattern of the hybrid shiverer-"myelin deficient" mice (Inoue *et al.*, 1988).

B. Demyelinating diseases of humans

In humans, the most prevalent and best known primary demyelinating disease is multiple sclerosis(MS) which affects over 50,000 people in Canada (150 individuals per 100,000 in Alberta) with, in genera a higher incidence in females than in males (Morell and Norton, 1980). Pathologically, MS is characterized by the presence of scarlike (sclerotic) plaques in which most, if not all of the myelin and the oligodendrocytes have been destroyed and replaced by astrocytes. The axons in the MS plaques are mostly spared and appear normal. Around the edges of each plaque, T_4^+ (helper, inducer) T cells and Ia^+ [class II major histocompatibility system (MHC) molecules, characteristic of antigen-presenting cells] macrophages, important in demyelination, have been observed (Traugott *et al.*, 1983). Myelin debris can be seen inside the macrophages in the

"active" plaques, those plaques where the destruction of myelin is still in progress (Epstein *et al.* 1983). The white matter surrounding the MS plaques usually appears normal.

The symptoms for MS vary; examples are visual disturbances, muscle weakness, lack of coordination and spasticity, all of which are due to blocked or unreliable conduction along nerves, a consequence of myelin loss. What induces the destruction of myelin in MS is still largely speculative. However, 2 classes of factors, environmental and genetic are usually considered in the aetiology of MS. The incidence of MS is known to vary with latitude, being higher as one goes further from the equato. in both the Northern and the Southern hemispheres. Attempts to correlate MS incidence with meteorologic and climatic effects (Kurland and Reed, 1964) including solar radiation and ultraviolet light radiation (Sharpe, 1986) are not conclusive. Migrations between high incidence and low incidence areas do to some extent affect MS distribution. For example, in Caucasians, migrations between northern Europe and South Africa (Kurtzke and Botha, 1970) or in the USA, between northern states and southern (Alter *et al.*, 1971; Kurtzke *et al.*, 1979) are associated with a reduction in the risk of MS if the moves occur in childhood before 15 years. However, migration after puberty will not change the disease risk of the migrants.

Stronger evidence for environmental factors involved in MS is given by the existence of clusters of cases or "MS-epidemics" at particular times and often in low incidence areas, the best example being in the Faroe Islands. The first MS cases in the Faroe Islands were reported in 1943, after the occupation by British troops; then between 1943 and 1960, 24 cases appeared (Kurtzke and Hyllested, 1979) and there has been only one further case since (Kurtzke, 1983; McDonald, 1986). Similar observations have been made in Iceland after both the First and the Second World Wars (Kurtzke *et al.*, 1980) and more recently in Macomer-Sardinia for the period between 1912 and 1981 (Rosati *et al.*, 1986). Because of these epidemics, the environmental MS factor was thought to be an infectious agent acquired in childhood. Spirochaetes have been suggested (Dick and Gay, 1988; Marshall, 1988) but theories for viral infections associated with MS are more commonly accepted despite the lack of evidence implicating a particular type of virus. Elevated serum and/or cerebro-spinal fluid (CSF) titers against measles, herpes, influenza, varicella and other viruses have been reported (Haire *et al.*, 1973; Johnson, 1982) but these are not specific and have often not been found consistently; similarly, the more recent reports implicating human T-lymphotropic retroviruses (Koprowski *et al.*, 1985) or Epstein-Barr viruses (Warner and Carp, 1988) have not been confirmed (Karpes *et al.*, 1986). In summary, there is

convincing evidence for an environmental factor in MS but the evidence as to its nature, possibly viral, is weak.

MS is not an inherited disease as such, however, several observations indicate some predisposition towards the development of MS. MS is rare or absent in some ethnic groups (Bantu, Eskimo, Orientals) irrespective of the latitude. The incidence of MS in Sapporo, Japan is 2 per 100,000 while is Boston which is at the same latitude (42° north) it is 41 per 100,000 (Kuroiwa *et al.*, 1983). The Hungarian gypsies are racially distinct from the Caucasians and only 2 cases of MS have been reported in the 110,000 Hungarian gypsy population; there are 37 MS cases per 100,000 for the Hungarian caucasians. Similarly in New Zealand, a high MS incidence area, with 69 cases per 100,000, the Maoris and the Asians seem genetically protected from developing the disease, only 1 MS case per 350,000 has been reported in these two groups (Miller *et al.*, 1986). The familial nature of MS is another indication for genetic components; the risk of acquiring MS is 5 to 25 times higher in first-degree relatives than in the general population (Wikström *et al.*, 1984). Even second- and third-degree relatives, who usually do not share a common environment, apparently have a slightly increased risk (Sadovnick and Macleod, 1981). In the study of twins, it appears that the risk for both members of a pair of twins to develop MS is higher if they are monozygotic than if they are not. In Canada, Dr. George Ebers and coworkers

(1986) have examined 5463 MS patients among which the incidence of twinning was 1 per 80 (the expected incidence in the general population). There was a total of 27 monozygotic and 43 dizygotic pairs. Seven of the 27 monozygotic pairs (i.e. about 26%) and only 1 of the 43 dizygotic pairs (2.2%) were concordant for MS. The concordance rate for 4582 non-twin sibling patients was 1.9%, close to the rate in dizygotic twins. These studies have provided the strongest evidence for a major genetic component in susceptibility to MS.

With respect to the nature of the genetic factor, an association has been found between MS and the HLA region, the region of chromosome 6 which is concerned with the genetic control of immune mechanisms. The strongest associations are with the DR loci and particularly the DR2 genes in populations of Northern European origin such as Scandinavians, North Americans, Australians and New Zealanders (Miller and Hornabrook, 1984; McDonald, 1984). In New Zealand, there is a low frequency of HLA DR2 antigens in the Maori population which has a very low MS incidence. However, the Hungarian Gypsies which are also relatively resistant to MS do have a high incidence of DR2 antigens, 57% as compared to 43% in the caucasian MS patients and 19% in the caucasian Hungarian controls (Palffy, 1982). In Arabs, MS is not associated with DR2 gene but rather with HLA DR4 gene (Kurdi *et al.*, 1977) while no HLA association has been

found in the Japanese or the Israelis (Naito *et al.*, 1982). The Shetland Islands have a high MS prevalence and also a high incidence of DR2 antigens but these DR2 antigens are found as frequently in MS patients as in the normal population (Compston, 1982). It is clear, therefore, that the DR2 gene cannot be the critical factor in conferring susceptibility to MS; it is neither necessary nor sufficient to lead to the development of the disease, but it could still be one of the many, unidentified yet, factors involved (Haile *et al.*, 1985; Salier *et al.*, 1986). Beside controlling susceptibility to the disease, genetic factors could probably affect its severity and perhaps explain the differences in the course of the disease with different patients: some patients may remain free of symptoms for years after a first attack, whereas others will suffer repeated relapses and die within 5 to 10 years. Concerning the origin and pathogenesis of MS, a popular hypothesis today is that of a slow virus infecting genetically susceptible individuals in their childhood; and later in life, there is an abnormal immune response against some of the white matter antigens causing destruction of myelin in the CNS.

The notion of autoimmune reactions directed against the CNS in MS has come from the study of experimental allergic encephalomyelitis (EAE), a neuroautoimmune disease which is considered as the animal model for MS. EAE, specifically, the chronic relapsing type, is pathologically and clinically

very similar to MS. EAE is induced in animals by the injection of whole brain tissue, myelin or myelin components along with adjuvants; it can also be produced by the transfer of lymphoid cells, mainly T cells, from an immune donor (Ben-Nun and Cohen, 1982; Pettinelli and McFarlin, 1981). Among the myelin components causing EAE, MBP is the most encephalitogenic and the T cells sensitized to MBP are those inducing chronic EAE in transfer experiments (Arnason, 1983). Although MBP-specific T cells have only been found in patients with postinfectious encephalomyelitis and not in MS patients (Hafler *et al.*, 1987), there are still indications that MBP is an important antigen in MS: anti-MBP antibodies are found in CSF of MS patients and their titer has been shown to increase dramatically during acute MS phase (Warren and Catz, 1986). Molecular mimicry is one of the mechanisms suggested to explain autoimmunization. In molecular mimicry the viral antigens resemble tissue components in such a manner that the immune response cannot differentiate between them and attacks directed against the virus will also affect tissue components. Of all the myelin antigens examined, MBP was the only one to show strong homologies with viral proteins. Fujinami and Oldstone (1985) have identified a 6 amino acid-peptide in Hepatitis B virus polymerase which is identical with a MBP sequence that is encephalitogenic in rabbits. Homologies between MBP encephalitogenic sequences (different MBP sequences are encephalitogenic in different animals) and sequences from human papovirus have been

reported (Jahnke *et al.*, 1985; Stoner *et al.*, 1986). It is therefore possible that, through molecular mimicry, infections with viruses also cause attack on MBP by the immune response and destruction of myelin.

MBP is probably not the only myelin component involved in MS pathogenesis. PLP, the major myelin protein has also been suggested as a target for immune destruction of myelin in MS (Tuohy *et al.*, 1988) and the PLP gene, which is located on the X chromosome (Hudson *et al.*, 1987) might be one of the factors responsible for the decreased MS susceptibility of males. There is also evidence for PLP inducing EAE in rabbits (Van der Veen *et al.*, 1986), guinea pigs (Yoshimura *et al.*, 1985), rats (Yamamura *et al.*, 1986) and mice (Trotter *et al.*, 1987). The other myelin components MAG, galactocere broside and other lipids, all appear to be unable, alone, to produce CNS disease; only in combination with MBP will MAG produce a mild monophasic EAE (Raine and Traugott, 1982).

In addition to its role in MS pathogenesis, MBP has also been used for diagnostic purposes and has been suggested for use in MS treatment. MBP is found at low levels in the CSF of MS patients and its levels increase during and immediately after an exacerbation of disease (Ohta *et al.*, 1980). CSF-MBP exist either free or bound to immunoglobulins G (IgG). Elevated levels of free CSF-MBP is characteristic of patients with active disease (Whitaker *et al.*, 1986) while increased

levels of IgG-bound MBP correlate with progressing disease (Warren and Catz, 1987). MBP is also found in circulating blood and urine of normal individuals, released during normal myelin turnover. Measurement of MBP levels in urine can be important to monitor MS activity since urine and blood MBP levels are known to increase in disease cases (Whitaker, 1987). There is no cure for MS but there have been many reports of MBP injection being effective in the treatment of EAE in animals (Levine and Sowinski, 1984; Higgins and Weiner, 1988). Based on these successes of EAE treatments, Eidinoff (1988) suggested that human MBP given intravenously together with immunosuppressive drugs and corticosteroid may suppress the autoimmune reaction in MS.

VI. DNA Polymorphism and Disease Association

The human genome is highly polymorphic: as many as one in every 300 base pairs differs in 2 comparable parental DNA sequences. Many of these differences are single base-pair changes which often introduce or remove a restriction site, or are sequence deletions, insertions or rearrangements, all of which affect the length of DNA between restriction sites, thereby creating restriction-fragment-length polymorphisms, RFLPs. Because RFLPs occur so frequently, every genetic disease is expected to be linked to and co-segregate with at least one RFLP (Watkins, 1988). RFLPs are therefore important genetic tools which have been extensively used in

the analysis and diagnosis of diseases. In some cases where the mutation which is responsible for the disease creates the RFLP, the presence of the RFLP allele itself signifies the disease state. Examples are in β-thalassaemia, where a deletion within the β-globin gene generates a diseased 3.7 Kb PstI allele instead of the normal 4.4 kb PstI fragment (Orkin *et al.*, 1978; Old *et al.*, 1982) . In the sickle cell anaemia, a loss of an MstII site (β^S allele) within the β-globin gene was observed (Geever *et al.*, 1981; Conner *et al.*, 1983). And in phenylketonuria, 200 base-pair insertions within the phenylalanine hydroxylase gene creates the diseased 4.2 kb or 4.4 kb HindIII RFLP alleles. The 4.0 kb HindIII band is the normal allele (Dilella *et al.*, 1985).

For other diseases, however, direct diagnosis is not possible because the precise mutation in the gene is not known; in these cases, family studies are necessary and diagnosis can be performed using an RFLP located outside the gene, but close enough to be linked to the disease. These indirect analyses are being frequently used in prenatal diagnosis of diseases like ornithine transcarbamylase deficiency (Rozen *et al.*, 1985), haemophilia A (Antonarakis *et al.*, 1985) and some phenylketonuria cases (Lidskey *et al.*, 1985). The risk of misdiagnosis in the indirect diagnostic approaches can be reduced substantially by using 2 RFLP markers, possibly flanking the disease gene (Wexler *et al.*, 1985). DNA polymorphisms have also been used to determine

the origin of some chromosomal imbalances and abnormalities. For example, in Down's syndrome, the trisomy of chromosome 21 is caused by nondysjunction, during meiosis in one parent; utilizing RFLPs it is possible to identify the parent who contributed the extra chromosome (Babu and Verma, 1986). In disease analysis, RFLPs have largely contributed to the elucidation of the mechanisms causing the pediatric tumors, retinoblastoma on chromosome 13 (Cavenee *et al.*, 1983) and Wilm's tumor on chromosome 11 (Koufos *et al.*, 1985). Where RFLP studies have had more impact is probably in the analyses of the ma inherited diseases of unknown aetiology, where the resp ible gene(s) is not known. RFLPs can be found associate ch a cloned DNA segment which is co-inherited with the disease. Following the inheritance of these cloned, "anonymous" DNA fragments in several large families has lead to the mapping of Huntington's disease to chromosome 4, six centimorgans (CM) or 6 x 10^6 base pairs away from one of the RFLP markers used (Gusella *et al.*, 1985; Folstein *et al.*, 1985); by *in situ* hybridization, the Huntington locus is now known to be between the chromosomal bands 4p16 and 4pter (Wang *et al.*, 1985). Similarly, the cystic fibrosis (CF) locus has been mapped to chromosome 7 using RFLPs on anonymous DNA fragments that are 10 CM (the paraoxonase, PON gene) (Wainwright *et al.*, 1985) and 15 CM (locus called Lam4-917) away (Tsui *et al.*, 1985). The CF locus maps in the middle of the long arm of the chromosome, close to the pro-α2(I) collagen gene on 7q21-7q22 area

(Scambler *et al.*, 1985). The RFLPs associated with CF are now used for carrier detection/exclusion and prenatal diagnosis of CF.

Other diseases mapped using RFLPs on anonymous DNA sequences include the adult polycystic kidney disease, 5 CM from the α-globin cluster on chromosome 16 (Reeders *et al.*, 1985), Becker muscular dystropy on the X chromosome (Brown *et al.*, 1985) and Wilson's disease which is linked to esterase D gene on chromosome 13 (Bonne-Tamir *et al.*, 1986). The genes responsible for chronic granulomatous disease (Royer-Pokora, 1986) and for Duchenne muscular dystrophy (Kunkel *et al.*, 1986), both on the X chromosome have been cloned without reference to specific proteins, just by relying on RFLP studies and chromosomal map positions; this is referred to as "Reverse genetics" (Francomano and Kazazian, 1986). An alternative to the use of anonymous DNA fragments is the study of RFLPs associated with candidate gene(s) (i.e. the genes that are likely to be involved because of the known pathophysiology or histologic characteristics of disease). If no linkage is found bewteen RFLPs around the candidate gene locus and the disease genotype, that candidate gene cannot be involved in the aetiology of the disease. The candidate gene approach excluded the T cell antigen receptor-β gene as a cause for ataxia telangiectasia (Berliner *et al.*, 1985), the apolipoprotein CII gene as a cause for hypercholesterolaemia (Donald *et al.*, 1985) and β-nerve

growth factor gene as the cause for neurofibromatosis (Darby *et al.*, 1985) or for familial dysautonomia (Breakefield *et al.*, 1984). The roles of α2(I) procollagen gene in type IV osteogenesis imperfecta (Grobler-Rabie *et al.*, 1986) and of rhodopsin gene in colorblindness (Nathans *et al.*, 1986) were confirmed by the candidate gene approach.

DNA polymorphisms can also be used in the analysis of non-genetic, multifactorial diseases provided a genetic factor affecting the risk (susceptibility, predisposition) is involved. The approach often used is to test for association between particular alleles of a polymorphism and the disease phenotype; usually a candidate gene for the disease is selected and a nearby RFLP is used as a tool to compare patients and controls in a general population. Positive associations between polymorphism and disease may sometimes be confirmed by additional family studies. Although these analyses are for the most part still preliminary, they have been successfully applied to some diseases as complex as atherosclerosis, cancers, breast tumors and non-insulin dependent diabetes mellitus (reviewed by Cooper and Clayton, 1988). Atherosclerosis is one of the most common multifactorial diseases which clearly shows a tendancy to cluster in families. Environmental factors increasing the disease risk include diet, smoking, excessive body weight and lack of exercise. However, minimizing these risk factors does not necessarily protect from atherosclerosis. Hegele

and Breslow (1987) have estimated that approximately 5% of the population in USA is protected from atherosclerosis and will never develop any disease regardless of their life-style, while another 10% of the population cannot escape from the disease. This indicates a strong genetic component involved in atherosclerosis and because of the relation between increased blood lipids and disease the apolipoprotein genes are possible candidate genes for disease susceptibility. Eleven RFLPs have been identified around the chromosome 11 apolipoprotein(apo-) genes and 6 of these RFLPs have been used in clinical studies. One of these RFLPs is a 300 base-pair deletion of an Alu element, the minor allele (Alu deletion) is more frequent in atherosclerosis patients than in normal controls (Frossard *et al.*, 1986); in another study, the presence of this minor Alu allele was associated with decreased mean levels of high density lipoprotein (HDL) cholesterol (Lim *et al.*, 1986). Elevated levels of low density lipoprotein (LDL) and depressed levels of HDL are strong predictors for atherosclerosis risk (Maciejkor *et al.*, 1983).

Another RFLP, due to a variant XmnI site around the apo-AI gene, reveals 2 bands, a major 8.3 kb and a minor 6.6 kb XmnI band. The frequency of the minor 6.6 kb XmnI allele was decreased in myocardial infarction patients (Hegele *et al.*, 1987) and in patients with coronary artery disease (Frossard *et al.*, 1986) when compared with normal controls. Some of

the RFLPs on the apo genes of chromosome 2 have also been clinically examined. One chromosome 2 RFLP generates 2 XbaI alleles, one of 8.6 kb and another with size 5.0 kb; both of these alleles are present with equal frequency in the general population and are, therefore, not associated with disease. The other apo-genes RFLPs on chromosome 2 are the 11 kb and 13 kb EcoRI minor and major alleles and 2 length variation alleles in A-T rich minisatellites, the longer allele, ID1 being the minor with the shorter allele ID2 being the more common allele. The frequencies of both the minor 11 kb EcoRI and the minor ID1 alleles were all significantly higher in cases of myocardial infarction than in controls (Berg *et al.*, 1985; Hegele *et al.*, 1986). None of these chromosome 2 apo-gene RFLPs affected the levels of LDL or HDL, suggesting that variation at this apo-gene locus may be a new, independent from blood lipid levels, risk factor for myocardial infarction. Many more RFLPs around other apo-genes (on chromosomes 1 and 19) have been studied and it appears that most of them are clinically useful in discriminating between cases of myocardial infarction and controls. In all these studies, the controls were usually sex- and age-matched but not often race-matched. Frequencies of RFLP alleles around some apo-genes have been shown to vary between racial groups (Ordovas *et al.*, 1986), an observation that might affect some of the disease association reported above. In cancer risk assessment, RFLPs around the Ha-ras gene have been found useful (Krontiris *et al.*, 1986); similarly, RFLPs within the

α1-anti-trypsin gene are associated with increased susceptibility to chronic lung disease (Kalsheker *et al.*, 1987).

In light of these reports, it seemed reasonable to undertake a study analysing the association between susceptibility to MS and RFLPs around the major myelin protein genes, particularly, the MBP gene. The purpose of this study was to clone sequences coding for human MBP which were not yet available. A comparison of these sequences (through hybridization patterns) from different individuals, healthy and MS patients would then establish first whether the MBP gene sequence showed DNA polymorphisms and second whether these polymorphisms could influence the risk of developing MS.

CHAPTER 2

MATERIALS AND METHODS

I. Preparation of Probes

A. Synthetic oligonucleotides

1. Chemical synthesis

Mixed oligonucleotide probes for MBP gene were synthesized by a phosphotriester method using the Bachem manual DNA synthesizer. All solvents and chemicals for DNA synthesis were purchased from BDH Chemicals Ltd. (Poole, England) and Aldrich Chemical Co. (Milwaukee, Wisconsin, U.S.A.). Synthesis was performed on a solid support, with the first nucleotide attached to polystyrene; elongation of the nucleotide chain occurred by condensation of charged, activated dimers. Activation of protected dimers involved initial removal of the 3' cyanoethyl protecting group using triethylamine then followed by coupling to the preceding dimer in the sequence (or to the support) and deprotection at the 5' terminus with removal of dimethoxytrityl (DMT) using zinc bromide. Coupling reactions were conducted in the presence of 0.3 M mesitylene sulfonyl nitrotriazole (MSNT), for 15 min with gentle agitation and the unreacted groups were capped by acetylation. The newly synthesized DNA fragments were cleaved from the resin support and most of the protecting groups removed by incubation at 45°C for 16-18 hr in the presence of 0.5 M tetramethylguanidinium-pyridine-aldoximate. Detritylations removing the 5' DMT group protecting the terminal nucleotide involved treatments with

80% (v/v) acetic acid for either 15 min for DMT-cytidine or -adenosine, 30 min for DMT-thymidine, or 40 min for DMT-guanosine. The synthesized oligonucleotides (25 and 27-mer) were sized and purified on 20% (w/v) polyacrylamide-7 M urea gels (Bio-Rad Laboratories, Richmond, CA., U.S.A.) described by Jing *et al.* (1986).

2. Labeling of oligonucleotide probes

Synthetic DNA probes (usually 0.5 µg) were labelled at their 5'termini with 50-100 µCi [γ-^{32}P]-ATP(3000 Ci/mmol) (Amersham Canada Ltd., Oakville, Ontario) using 10 units of T4 polynucleotide kinase (Pharmacia P-L Biochemicals Inc., Dorval, Quebec) in buffers containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 1 mM EDTA. Reactions were carried out at 37° C for 2 hrs and the kinase was inactivated by incubation at 65°C for 10 min. Unincorporated nucleotides were removed by gel filtration chromatography on Sephadex G-25 columns (Pharmacia P-L Biochemicals). Final specific activities varied between 0.5 and 1.5 x 10^8 cpm/µg.

B. C-DNA probe

The cloned MBP c-DNA probe was derived from plasmid pMBP-1, a generous gift from Dr. L. Hood (Caltech, California). Plasmid pMBP-1 is a pBR322 derivative containing, at the EcoRI site, a 1.45 kb insert which is the

c-DNA containing the complete coding region for the small 14.0 kDa rat MBP (Roach *et al.*, 1983).

The plasmid was propagated in *E. coli* strain HB101 in the presence of 40 µg/mL ampicillin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and purified by equilibrium centrifugation in a cesium chloride (Terochem Laboratories, Ltd., Edmonton, Alberta) - ethidium bromide gradient (Sigma Chemical Co.) as described by Clewell and Helinski (1972). Electrophoresis in low melting point agarose (Bethesda Research Laboratories, Burlington, Ontario) was used for recovery and purification of the c-DNA insert.

The c-DNA probe was labeled by nick-translation, using the nick-translation kit supplied by Boehringer-Mannheim Biochemicals (Dorval, Quebec) and [α-^{32}P]-dCTP (>3000 Ci/mmol) (Amersham), to final specific activities of 1-3 x 10^8 cpm/µg.

II. Sample Collection

A. Placentae

Human placental samples were obtained from Dr. L. H. Honore (Department of Pathology, University of Alberta, Edmonton, Alberta) and from the maternity ward of the University of Alberta Hospitals. The medical histories of the placenta donors were not available.

Fresh placental tissue was immediately cut in small pieces, frozen with liquid nitrogen and then ground to fine particles. The powdered tissue was kept at -70°C until DNA extraction.

B. MS patients

Thirty-four patients (17 men and 17 women) were selected through the multiple sclerosis clinic (University of Alberta Hospitals) and clinically diagnosed as exhibiting definite MS symptoms by Dr. K. Warren. Diagnosis of definite MS, chronic progressive or relapsing/remitting types, was based on established criteria (Shumacher *et al.*, 1965; Rose *et al.*, 1976).

C. Controls

Thirty four healthy individuals were selected by Dr. K. Warren as age, sex and race-matched controls for the MS patients.

Blood samples were also obtained from 10 randomly chosen individuals, male and female, aged between 25 and 45 and from different race

s. None of these 10 individuals had any known personal or family history of MS.

III. Library Screening

A c-DNA library for human brain was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). This library contained approximately 700,000 independent λgt11 phages, growing in *E. coli* strain Y1090. Plating Y1090 bacteria for phage growth were prepared according to Maniatis *et al.*

(1982). Briefly, the bacteria were cultured for 12 hr in Luria broth [10 g/L bacto-tryptone (Difco Laboratories), 5 g/L bacto-yeast extract (Difco Laboratories), 10 g/L NaCl]. Screening of the library was carried out in 3 major steps. The phages were initially plated on large 150mm petri dishes containing 1.5% (w/v) bottom agar (Difco Laboratories, Detroit, Michigan, U.S.A.). After 12 hr of growth at 37°C, the plaques were transferred onto 137 mm sterile nitrocellulose filters (New England Nuclear Products, Boston, MA, U.S.A.) and amplified for an additional 12 hr, *in situ*, sandwiched between two nitrocellulose filters as outlined by Maniatis *et al.* (1982). Up to 50,000 plaques were screened on each filter. Positive plaques were picked from the original plate and phage particles allowed to diffuse out of the agar by incubation at room temperature for 2 hr in SM buffer [0.01% (w/v) gelatin (Difco Laboratories), 100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl (final concentrations; the Tris-HCl was diluted from a 1M stock solution, pH 7.5 at 22°C)], One plaque yielded 10^6-10^7 phage particles. The second screening was performed after amplification of phages in liquid, Luria broth cultures on 96-well microplates. Briefly, 25-50 µL of bacterial culture were mixed with 50-100 µL of phage in a microplate well; following a 20 min period incubation at 37°C to allow adsorption of phages to bacteria, 150-200 µL of Luria broth was added and the plate was incubated with vigorous shaking at 37°C until complete bacterial lysis had occurred. Bacterial lysis was often

completed by addition of 3 µl of chloroform and further incubation at 37°C for 30 min with vigorous shaking. Phage samples, 10-20 µL (approximately 10^6 particles) were subsequently spotted onto nitrocellulose paper (Schleicher and Schuell, BA85 0.4 mm) for further screening. The positive phages from microplates were directly plated onto small 85 mm petri dishes and grown at lower densities (approximately 3000 plaques/dish) so as to have clear, individual plaques generated by single phages.

For each screening, the filters containing phages were denatured for 5-10 min in 0.5 M NaOH-1.5 M NaCl, neutralized for 10 min. in 0.5 M Tris-HCl (pH 8.0 at 22° C)-1.5 M NaCl then baked for 2 hr at 80°C. Prior to hybridization, the filters were immersed in 6 x SSC (20 x SSC = 3 M NaCl, 0.3 M sodium citrate) for 5 min at room temperature and prewashed at 42°C for 2 hr in a solution containing 0.05 M Tris-HCl (pH 8.0), 1 M NaCl, 0.001 M EDTA, 0.1% (w/v) sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories). Prewashing was necessary to remove fragments of agarose and bacterial debris.

IV. DNA Preparation

A. Lambda phage DNA

Recombinant λgt11 phages were prepared using a modification of the plate lysate method outlined by Maniatis *et al.* (1982). Plaque forming units (Pfu's) of phage (1-5 x 10^6) were mixed with 300 µL of Y1090 plating bacteria and

incubated at 37°C for 20 min before spreading on 150 mm fresh Luria broth-agar plates. After growth at 37°C for 12 hr, the plates were flooded with 10 mL of SM buffer and stored at 4°C for 4-5 hr with intermittent gentle shaking. The SM was harvested, the plates flooded again with 5 mL of SM and stored for 15 min in a tilted position to allow the fluid to drain into one area. The harvests of SM were combined and centrifuged at 5000 rpm for 10 min to pellet bacteria and agar; supernatants were then centrifuged in ultraclear tubes in a SW27 (Beckman, Palo Alto, CA) rotor at 23000 rpm, for 1.5 hr at 4°C. Phage plates were resuspended in 2 mL of SM by gentle shaking for 5 hr at 4°C and mixed with 0.75 g/mL solid cesium chloride before centrifuging in ultraclear tubes using the SW40 rotor at 35000 rpm for 24 hr at 4°C. The band of phage particles was collected, the cesium chloride was removed and the phage DNA purified using proteinase K treatment (Sigma Chemical Co.) followed by sequential extractions with phenol equilibrated with a 1/10 volume of TE buffer [10 mM Tris-HCl, 1 mM EDTA (final concentrations; the Tris-HCl was diluted from a 1M stock solution, pH 8.0 at 22°C] , phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) as described by Maniatis *et al.* (1982).

The c-DNA inserts containing MBP sequences (average size 900 base-pairs) were recovered from recombinant phage DNA by digestion with EcoRI and electrophoresis in low melting point agarose gels. The agarose slice containing the DNA band was

excised. After adding 5 volumes of TE buffer, the agarose was melted by incubation at 65°C for 10 min and the DNA was purified by two phenol extractions and one ether extraction. The DNA was alcohol-precipitated in the presence of 2 volumes of 95% ethanol and a 1/10 volume of 3 M sodium acetate (pH 7.0 at 22°C), collected by centrifugation and resuspended in TE buffer.

B. Placental DNA

The procedure used to prepare high molecular weight placental DNA was a modification of the protocol described by Blin and Stafford (1976). The ground placental tissue was mixed with 5 volumes of 0.5 M EDTA (pH 8.0 at 22°C), 0.5% (w/v) lauryl sarkosyl, 1 mg/mL pronase (Sigma Chemical Co.) (pretreated by self-digestion for 2 hr at 37°C) and incubated at 37°C for 2 hr; one volume of 0.5 M EDTA (pH 8.0 at 22°C), 0.5% (w/v) lauryl sarkosyl, 100 µg/mL of proteinase K was then added and the incubation continued, at 50°C, for an additional 2 hr. The viscous solution was swirled periodically during the 4 hr protease digestion. Proteins were removed by gentle extractions with equal volumes of phenol. After three phenol extractions, the DNA was dialyzed against a solution of 50 mM Tris-HCl (pH 8.0 at 22°C), 10 mM EDTA, 10 mM NaCl, with several changes. RNA was removed by treatment with 100 µg/mL heat-treated, DNase-free RNase (Sigma Chemical Co.) at 37°C for 3 hr followed by two extractions with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and extensive dialysis against TE buffer.

The purified DNA was precipitated by ethanol into long strands of high molecular weight DNA that were subsequently dissolved overnight on a rotator at 4°C. Aliquots of the purified DNA were run on 0.5% agarose gels (Bio-Rad Laboratories, Richmond, CA., U.S.A.) to verify that the DNA size was greater than the 50 kb intact lambda DNA.

C. Peripheral blood DNA

Whole blood (14 mL) was collected in EDTA-containing vacutainer (purple-stoppered) tubes and DNA was extracted exactly as outlined by Dr. D. Hoar (Madisen *et al.*, 1987). Briefly, the non-nucleated blood cells were lysed by osmotic shock with ammonium chloride while nucleated cells were lysed using SDS solutions containing EDTA. Protease digestion was omitted to facilitate digestion of DNA by restriction enzymes (Hoar , 1987), proteins were removed by phenol followed by chloroform extractions and DNA was precipitated, free of RNA, using salt and isopropanol.

V. DNA Hybridization Analysis

A. Restriction endonucleases

The restriction enzymes were obtained from Pharmacia P-L Biochemicals, Bethesda Research Laboratories and New England Biolabs (Beverly, MA., U.S.A.). Digestions were carried out using buffer conditions recommended by the suppliers with 2-3 enzyme units/µg of DNA, in volumes of 50-100 µL. When necessary, the volumes of reaction mixtures were reduced before gel loading by precipitation with ethanol in the presence of 5 M ammonium acetate, in a dry-ice/methanol bath.

B. Agarose gel electrophoresis

Electrophoresis of DNA was carried out in agarose gels ranging from 0.5 to 2% agarose in 1 x TBE buffer [50 mM Tris-borate (pH 8.3 at 22°C), 1 mM EDTA] at constant current. DNA fragments were visualized by staining with 0.5 µg/mL ethidium bromide. The sizes of the fragments were determined by comparing their migrational positions with those of the molecular size DNA markers: lambda phage DNA cut with HindIII or AvaI.

C. DNA transfers

Blotting of electrophoretically separated DNA fragments was performed by the method of Southern (Southern, 1975) as modified for transfers onto Genescreen Plus membranes (New England Nuclear Products). The DNA was often acid nicked by incubating the gels in 0.25 N HCl for 15 min at room temperature, prior to alkaline treatment. Transfers were carried out for 36 hr and the Southern filters immersed in 0.4 N NaOH for 60 sec, then in 0.2 M Tris-HCl (pH 7.5 at 22°C) - 2 x SSC as recommended by New England Nuclear Products. The filters were baked at 80°C for 1.5 hr before hybridization.

D. DNA hybridization

The dry filters were initially soaked in 2 x SSC for 15 min at room temperature.

For hybridization of DNA attached to nitrocellulose paper, the filters were prehybridized for 24 hr at 42°C in a solution containing 50% (v/v) deionized formamide (Terochem

Laboratories Ltd.), 5 x Denhardt's solution [0.1% (w/v) each of Ficoll, polyvinylpyrollidone-360 and BSA (Sigma Chemical Co.)], 5 x SSPE (x SSPE = 3.5 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA (pH 8.0 °C)], 0.1% (w/v) SDS and 100 µg/mL of heat denatured salmon sperm DNA (Sigma Chemical Co.). The hybridization buffer was identical to the prehybridization solution except for the use of 1 x Denhardt's solution, 0.3% (w/v) SDS and addition of 10% (w/v) sodium dextran sulfate (Pharmacia P-L Biochemicals) in hybridizations with nick-translated probes. Hybridization was carried out for 24-36 hr at 42°C with radiolabeled probe at concentrations of approximately 1-2 x 10^6 cpm/mL. Filters which had been hybridized with synthetic oligonucleotide probes were washed utilizing 5 x SSC, first at room temperature for 10 min. The buffer was changed and washing repeated for 4 to 5 times. Then the washings were performed at higher temperatures, between 55 and 68°C, twice for 60 min each. The washing temperatures were usually 2°C below melting temperatures of the synthetic probes as calculated by assuming a 2°C melting temperature for each adenine-thymidine pair and 3°C for melting a guanine-cytidine base pair. Nitrocellulose filters which had been hybridized to c-DNA probes were washed 4 times for 15 min each, at room temperature using 2 x SSC - 0.1% (w/v) SDS, followed by two washes for 60 min each at 60°C in 1 x SSC - 0.1% (w/v) SDS and 0.2 x SSC - 0.1% (w/v) SDS, respectively.

Genescreen Plus filters were prehybridized for at least 3 hr in a solution containing 50% (v/v) deionized formamide, 1 M NaCl, 1% (w/v) SDS, 10% (w/v) sodium dextran sulfate, 250 µg/mL of heat denatured salmon sperm DNA. Then the hybridization was performed for 36 hr at 42°C in the same buffer. Blots were washed twice, for 30 min. each, at 65°C in 2 x SSC - 1% SDS followed by two, 30 min washes at room temperature with 0.1 x SSC.

Filters were blotted dry and autoradiography performed using Kodak x-omat AR5 films (Eastman Kodak Co, Rochester, N.Y., U.S.A.) with one intensifying screen (Dupont Lightenng Plus), for 1-3 days at -70°C.

VI. Statistical Analysis

Chi-square tests of significance were used to analyze the fit between the observed and the expected polymorphic genotype frequencies. Chi-square (χ^2) values $\chi^2=\sum_i \frac{(o_i - e_i)^2}{e_i}$, likelihood ratio (G) (Sokol and Rohlf, 1983):

$$G = 2\sum(obs)\times\ln\frac{(obs)}{(exp)}$$

and proportion test values (z) were calculated for appropriate degrees of freedom [df = (rows-1) (columns-1)] and compared to values from R x C contingency tables (Mourant *et al.*, 1978).

The risk factor or relative incidence (RI) was calculated as follows:

$$RI = \frac{\text{(patients with tested genotype)} \quad \text{(controls with normal genotype)}}{\text{(controls with tested genotype)} \quad \text{(patients with normal genotype)}}$$

CHAPTER 3

ISOLATION OF SEQUENCES CODING FOR HUMAN MBP

I. Introduction

At the outset of this study, there was no information available on the human MBP gene, although the amino acid sequence of the 18.5 kDa human MBP had been published in the 1970's (Carnegie, 1971). Studies on other animal MBP genes were initiated with the cloning of a c-DNA encoding the small rat MBP (Roach *et al.*, 1983) using primarily information from protein sequences (Martenson *et al.*, 1972; Dunkley and Carnegie, 1974). Briefly, a stretch of 5 amino acids was selected from the rat MBP sequence and was reverse-translated into all the possible combinations of nucleotides. A total of sixteen 14-mers were synthesized and their mixtures were utilized to screen a c-DNA library from 18-day-old rat brain. Synthetic tetradecamer oligonucleotide probes based on amino acid sequences have also been used to isolate the c-DNA's coding for mouse MBP's (Zeller *et al.*, 1984).

Similar strategies were used in this study in an attempt to clone human MBP genomic sequences. Because complex genomic DNA's rather than c-DNA libraries were being screened, longer, 25 and 27-mer nucleotide sequences were synthesized.

II. Results

A. Attempts to clone human MBP genomic sequences

In the human 18.5 kDa MBP, the amino acids from positions 9 to 17, 88 to 96 and 137 to 145 represent the longest stretches of 9 or 10 amino acids in the amino-terminus, middle portion and in the carboxy-terminus, respectively, with minimal degeneracy in the amino acid code. These stretches of amino acids were also conserved between mouse, rat and human. These regions were, therefore, selected for reverse translation of their amino acids into nucleotides as shown in Fig. 2. The nucleotide sequences corresponding to all of the combinations coding for $_{9}$Gln-Arg-His-Gly-Ser-Lys-Tyr-Leu-Ala$_{17}$ (probe A), for amino acids $_{87}$Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro$_{96}$ (probe B) and for amino acids $_{137}$Ala-His-Lys-Gly-Phe-Lys-Gly-Val-Asp$_{145}$ (probe C) were chemically synthesized and purified. Aliquots of each of the synthetic nucleotide mixtures were end-labelled with T4 polynucleotide kinase and γ-^{32}P-ATP and their size was verified by running on polyacrylamide-urea gels. The autoradiogram of Fig. 3 showed that the nucleotide mixtures were of the proper size, *i.e.* 25 bases (probe A) and 27 bases(probes B and C). The size marker shown beside the synthetic nucleotides' lanes is a continuous sequence ladder of 1-base intervals that covers a size range from 12 bases on the bottom to more than 50 bases in length. This size marker is a mixture of T4 polynucleotide kinase-labeled and terminal deoxynucleotidyltransferase-labeled oligo(dT)$_{12-18}$. Because

Fig. 2. Sequences of the mixed oligonucleotide probes derived from the amino acid sequence of human MBP. The peptides selected from human MBP for reverse translation into nucleotides are presented with the nucleotide sequences corresponding to all the possible coding combinations. Probe A was derived from amino acids 9 to 17, probe B from amino acids 87 to 96 and probe C from amino acids 137-145.

Probe A, 25-mer

NH_2....Gln - Arg - His - Gly - Ser - Lys - Tyr - Leu - Ala....COOH

```
5'.... AA - CGA - CAC - GGA - TCA - AAA - TAC - CTA - GC.....3'
       G    G     T     G     G     G     T     G
            C           C     C                 C
            T           T     T                 T

          AGG             AGC                 TTA
            A               T                   G
```

Probe B, 27-mer

NH_2....Val - His - Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro....COOH

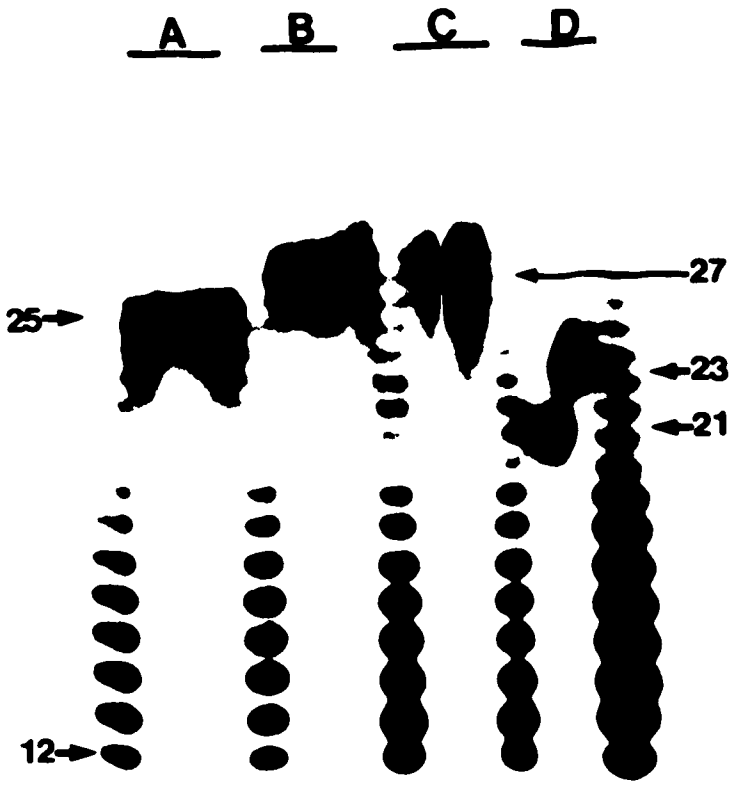
```
5'.....A - CAC - TTC - TTC - AAA - AAC - ATA - GTA - ACA - CC.....3'
       G     T     T     T     G     T     C     G     G
       C                                   T     C     C
       T                                         T     T
```

Probe C, 27-mer

NH_2....Ala - His - Lys - Gly - Phe - Lys - Gly - Val - Asp....COOH

```
5'....GCA - CAC - AAA - GGA - TTC - AAA - GGA - GTA - GAA....3'
       G     T     G     G     T     G     G     G     G
       C                 C                 C     C     C
       T                 T                 T     T     T
```

Fig. 3. Estimation of molecular size of the mixed oligonucleotides synthesized by the manual DNA synthesizer. Lanes A, B and C contain the synthetic oligonucleotides probe A (25-mer), probe B (27-mer) and probe C (27-mer) respectively; lane D contains 21-mer and 23-mer synthetic fragments used as molecular size standard; interspersed between lanes A, B, C and D are ladders of size markers (12 to 35 bases) containing a mixture of T_4 polynucleotide kinase-labeled and terminal deoxynucleotidyl transferase-labeled oligo $(dT)_{12-18}$.

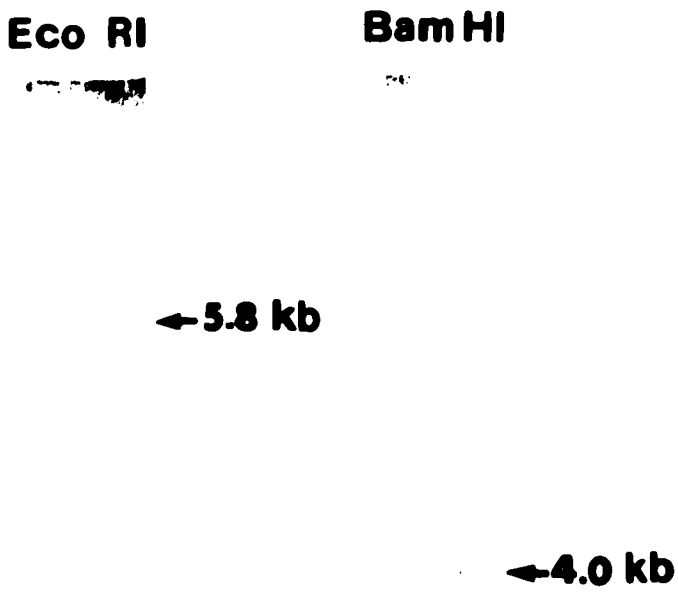

A
B
C
D
27
25
23
21
12

the synthetic oligonucleotides were synthesized as mixtures rather than individual sequences, their nucleotide sequences were not verified. However, the two control nucleotide fragments 21-mer and 23-mer shown in Fig. 3 had the proper nucleotide sequences when verified by DNA sequencing (data not shown). These 21 and 23-mer fragments were synthesized under the same conditions as the nucleotide mixtures A, B and C.

Placental DNA was digested with either restriction endonuclease EcoR1 or BamH1. The resulting fragments were fractionated by electrophoresis on agarose gels, then Southern blotted and hybridized to the synthetic probes A, B or C (Fig. 4). After several trials to adjust washing conditions, only probe B gave reasonably satisfying results. Positive hybridizing bands were observed at 5.8 kb with EcoRI digests or at approximately 4.0 kb with BamH1 digests. These hybridization bands were faint but readily reproducible. Probes A and C showed weaker hybridization signals which were often too difficult to visualize due to stronger backgrounds (data not shown). Attempts (temperature, salt, hybridization time) to intensify the hybridization bands with probe B and reduce the background in hybridization to probes A and C were unsuccessful.

The synthetic oligonucleotide probe approach might not be an adequate method to isolate MBP genomic sequences but interesting information was still obtained: the 5.8 kb EcoRI and 4.0 kb BamH1 bands must be parts of the middle portion of

Fig. 4. Hybridization of human placental DNA with mixed synthetic probe B. Placental DNA was digested with restriction endonucleases EcoRI or BamHI then Southern blotted and hybridized to 0.5 µg end-labeled mixed oligonucleotide probe B. The hybridized fragments and their sizes are indicated beside each autoradiogram.


Eco RI
Bam HI
5.8 kb
4.0 kb

the human MBP gene and must contain sequences corresponding to exon 3 and exon 4 (Roth *et al.*, 1987).

B. Isolation of c-DNA clones for human MBP

A c-DNA library of human brain containing approximatively 700,000 clones was screened using the MBP c-DNA from rat, isolated by Roach and coworkers (1983). Approximately 400,000 plaques grown on large 150 mm dishes were screened using the *in situ* filter amplification described in Materials and Methods. Fig. 5 shows representative filters with small λgt11 plaques hybridized to rat MBP sequences; 12 dishes were screened and 300 to 350 plaques were positive, of which 250 were transferred onto microplates. After amplification on microplate wells, the plaques (estimated to 10^6 phage particles) were spotted on and their DNA bound to nitrocellulose filters for hybridization. As seen in Fig. 6, a large number of phages did hybridize to MBP sequences. However, some wells (e.g. plate 2, row A, well 1 or well 9) gave stronger hybridization than others (plate 3, row A, well 7). To exclude the possibility that these differences in hybridization intensity resulted from differences in the number of phages present in the wells, phage titers for samples in microplate wells were compared. Results in Table I show that the hybridization intensity was not necessarily related to phage titer but instead reflected the presence of MBP-positive phage DNA. For example, there were more phages in microplate 3, row D, well 39 (underlined in Fig.6, plate 3) which did not

Fig. 5. Screening of the λgt11 c-DNA library of human brain with a c-DNA probe of rat MBP. A representative autoradiogram derived from 150 mm nitrocellulose filter containing MBP-positive phages. Arrows indicate some of the MBP-positive plaques (30-40 per filter) collected from petri-dishes, for further screening.

Fig. 6. Screening for MBP-positive phages among λgt11 phages amplified on microplate wells. Phage samples growing on 3 microplates were dot blotted on nitrocellulose filters in the same order as in the microplates and hybridized to the rat MBP c-DNA probe. Plate 3 rows G and H were negative controls with row G containing λ phages lacking MBP sequences and row H containing SM buffer only. The samples underlined showed different hybridization intensity: plate 3 row D was negative, plate 3 row A was weak, plate 2 row A showed a strong hybridization signal and plate 1 row A showed intermediate to strong hybridization signal.

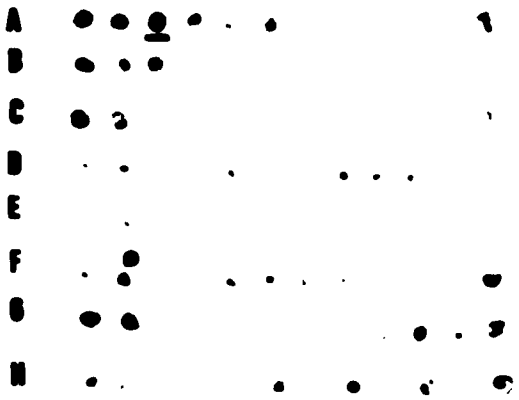


Plate 1

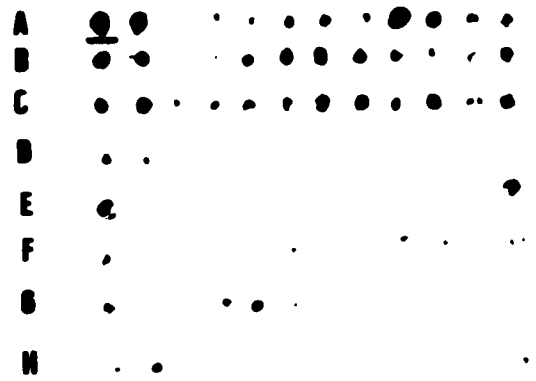


Plate 2

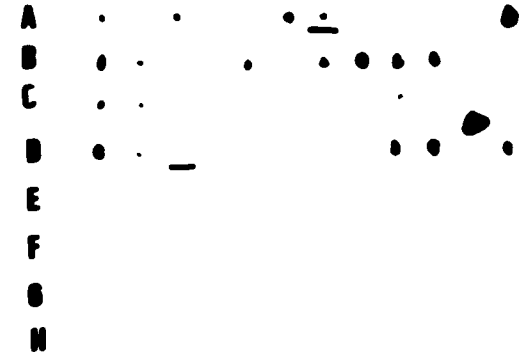


Plate 3

Table 1. Titer of phages from microplate wells.

	Hybridization Intensity	Phage titer Pfu/ml
Plate 1 - row A	++	1.1×10^9
Plate 2 - row A	+++	6.8×10^9
Plate 3 - row A	+	1.5×10^9
Plate 3 - row D	-	2.5×10^9

The plates (1, 2, 3) are the 3 microplates shown in fig. 6: phage samples with different hybridizatic intensity were picked from the wells underlined in fig. 6.

Each "+" indicates positive hybridization, ranging from + (weak) to +++ (strongest).

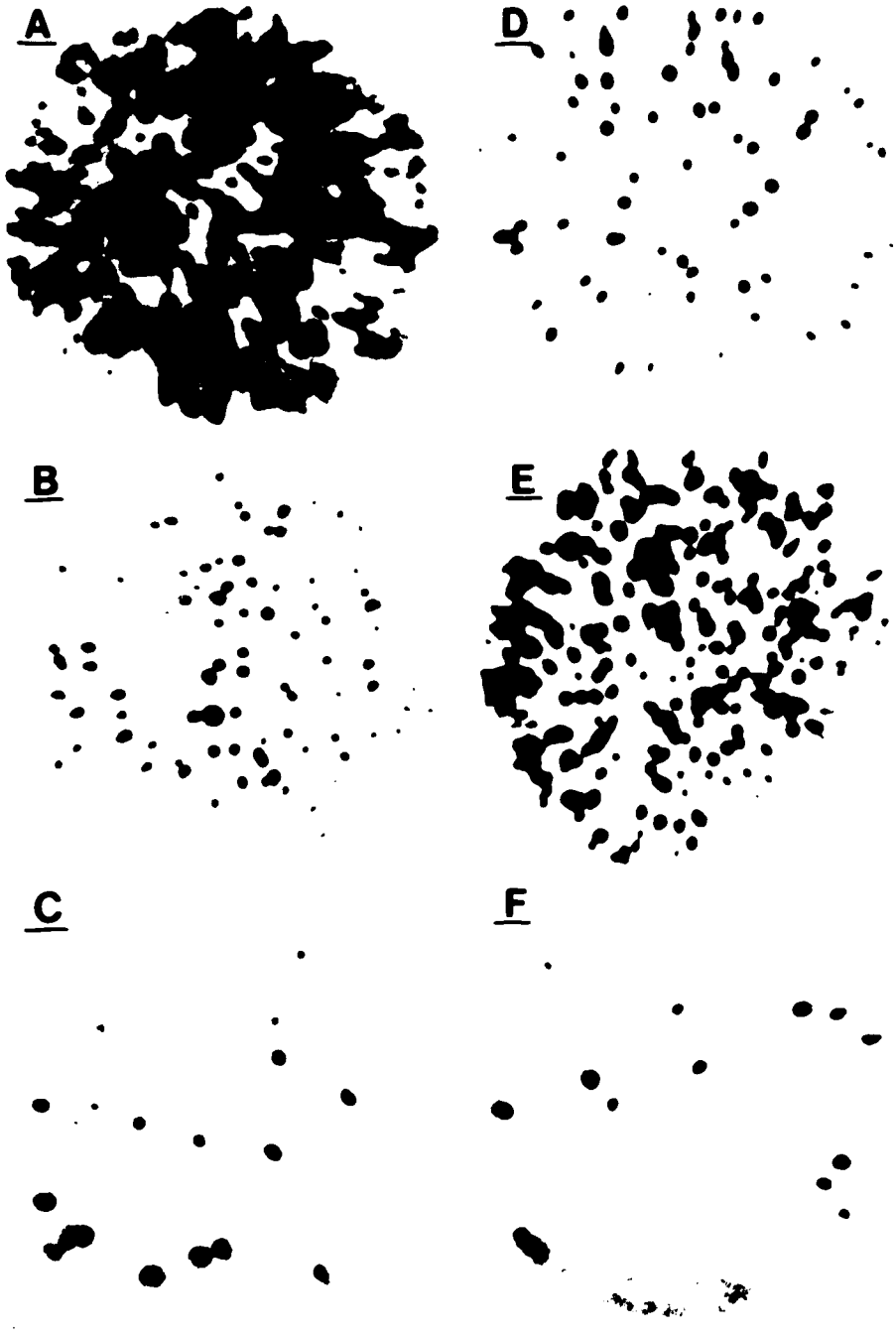
The "-" indicates no hybridization.

hybridize at all, than in row A, well 3 of microplate 1 (Fig. 6) which hybridized strongly. Also row A, well 3 of microplate 1 in Fig. 6 contained approximately the same number of phages as in row A, well 7 of microplate 3 which showed a weaker hybridization signal. Negative controls, rows C and H of microplate 3 consisted of either phages from petri dishes that did not hybridize in the first library screening step (row G) or simply of SM buffer plus Luria broth (row H).

In the third step of library screening, strongly MBP-hybridizing phages from the microplate wells of Fig. 6, were grown on small petri dishes at low enough densities (~3000 plaques per dish) so that the plaques appeared distinct and well separated from each other. The petri dishes that were screened all contained approximately the same plaque density before transfer to nitrocellulose filters and hybridization. The autoradiogram in Fig. 7 showed some of the filters with phages grown on small petri dishes. A comparison of filter A or filter F containing several positive hybridizing plaques with filter C or filter F which only had a few MBP-positive hybridizing plaques indicated the screening for MBP-positive phages gave genuine signals.

Twenty-four recombinant phages were picked randomly among the positive plaques in small petri dishes and lambda phage DNA was prepared from each of them. The DNA was then digested with EcoRI, the cloning enzyme used in the preparation of the library and the presence of a c-DNA insert

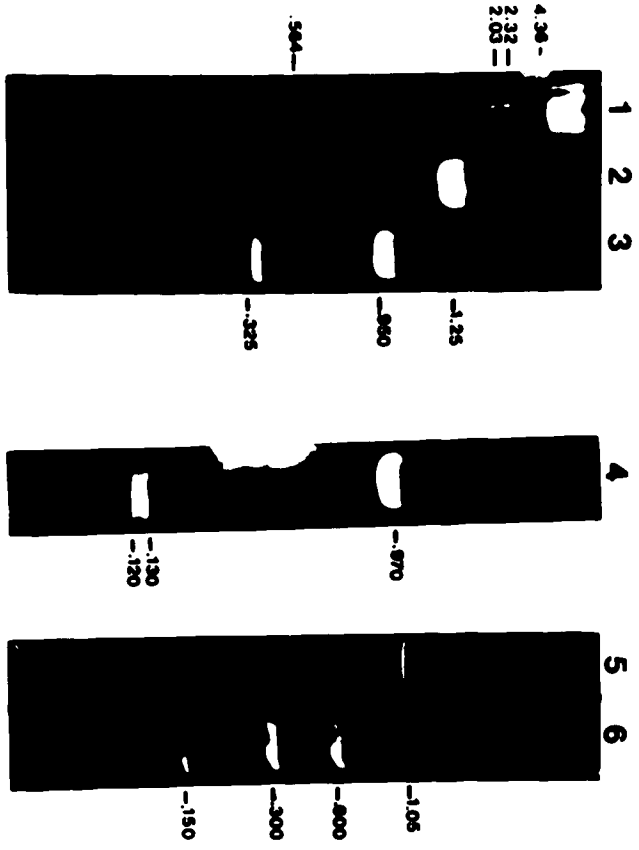
Fig. 7. MBP-positive hybridizing phages from a λgt11 c-DNA library of human brain. Phages were grown on 85mm petri dishes and the plaques from dishes containing approximately 4000 plaques each, were transferred onto nitrocellulose filters for hybridization to the rat MBP c-DNA probe.


A
D
B
E
C
F

was monitored by agarose gel electrophoresis. DNA inserts of different sizes, ranging from 600 base pairs (bp) up to 1200-1300 bp were obtained; all of them hybridized to the rat MBP c-DNA probe, some more intensely than others (data not shown).

Confirmation of the identity of the c-DNA inserts was greatly facilitated by publications of the complete sequence of the human MBP c-DNA from 2 different groups, while this work was in progress. Sequencing was, therefore, not necessary and the sequence of the purified c-DNA inserts was simply verified by restriction mapping based on the published human MBP c-DNA sequence (Roth *et al.*, 1986; Kamholz *et al.*, 1986). The larger c-DNA inserts obtained in this study were approximately 1.2 to 1.3 kb in size (Fig. 8, Lane 2) and hybridized strongly to rat MBP sequence. Digestion of this 1.3 kb EcoRI c-DNA insert with BamH1 did not change the fragment size on a 1.5% agarose gel while digestion with PstI (Fig. 8, Lane 3) generated 2 fragments with size approximately 950 bp and 300-350 bp. This 1.3 kb c-DNA insert contained 2 PvuII sites since 3 fragments of size 970 bp, 130 and 120 bp were generated upon digestion with PvuII (Fig. 8, Lane 4). The human c-DNA for MBP is 2.2 to 2.3 kb long and is cleaved by EcoRI into 2 fragments of 1.3 kb and 1.0 kb. The 1.3 kb EccRI portion of the c-DNA contains a single BamH1 site, 50 to 60 bp from the 5' end; 1 PstI site is present between nucleotide pairs 950 and 1000, close to a

Fig. 8. Restriction enzyme analysis of 2 MBP-positive hybridizing DNA inserts purified from c-DNA library of human brain. The c-DNA inserts were recovered from low melting point agarose gels of recombinant phage DNA digested with EcoRI, as outlined in Materials and Methods. The larger c-DNA insert, shown in lane 2, was digested with restriction endonucleases PstI (lane 3) or PvuII (lane 4). The second c-DNA insert (lane 5) was digested with XbaI (lane 6). The sizes of the DNA fragments, shown in kilobases, were determined by comparison of migration rates with the marker DNA fragments: λ phage DNA cut with HindIII (lane 1).


4.36
2.32
2.03
.564
1
2
3
.325
.960
1.25
4
.130
.120
.570
5
6
.150
.300
.600
1.05

PvuII site; a second PvuII site is found toward the 3' end of the c-DNA insert, approximately 150 bp from the EcoRI site (Kamholz *et al.*, 1986). The 1.3 Kb EcoRI c-DNA insert isolated was therefore identical to the 1.3 Kb EcoRI fragment of the human MBP c-DNA, the fragment which contains the coding region.

Two of the c-DNA inserts purified here were about 1.0 kb; one of them must correspond to the 1.0 kb EcoRI portion of the MBP c-DNA (Fig. 8, Lane 5) since it contained no BamH1, BglII, HincII, PstI or PvuII sites but was cleaved twice by XbaI into fragments with size 650 bp, 300 bp and 150 bp (Fig. 8, Lane 6). This 1.0 kb c-DNA insert showed considerably weaker hybridization to rat MBP sequences than the 1.3 kb insert (data not shown).

III. Discussion

The synthetic oligonucleotide approach (reviewed by Itakura *et al.*, 1984) is often the method of choice for cloning DNA sequences encoding proteins for which partial amino acid sequences are known. Although DNA sequences coding for small proteins (Suggs *et al.*, 1981), rare proteins (Ullrich *et al.*, 1983) and low copy number DNA sequences (Goeddel *et al.*, 1980) have been isolated, abundant DNA sequences are more easily cloned by the synthetic oligonucleotide method. At the outset of this work, it was not known whether the human MBP gene existed as a single copy or in multiple copies. It has now been established that it

is a single gene, 30 to 32 kb long with 7 small exons separated by very long introns (Roth *et al.*, 19[illegible]; Boylan *et al.*, 1986). Low representation of the MBP gene in the complex human genome, together with the fact that the hybridization probes used were exon-specific and relatively short (25 to 27 nucleotides) may explain the weak hybridization signals (Fig. 4). The chance of cloning MBP genomic sequences by this technique would certainly be improved if, rather than screening whole genomic DNA, a chromosome-18-specific (MBP gene maps on chromosome 18) library was available. In fact mixed synthetic probes have only been used successfully in screening c-DNA libraries or libraries enriched in the specific cloned genomic DNA (Montgomery *et al.*, 1978; Wallace *et al.*, 1981).

Complexity of the mixtures of oligonucleotide sequences might explain some of the hybridization problems (strong background, difficulty in finding appropriate washing temperatures) since probe mixture B, the probe that generated the strongest signals (Fig. 4) showed the least codon degeneracy and consequently contained less oligonucleotide sequences than the other 2 probes (Fig. 2). However, oligonucleotide mixtures as complex as 96 twenty-mer (Gubler *et al.*, 1983) and 384 twenty three-base long sequences (Whitehead *et al.*, 1983) have been used successfully.

The high degree of homology (over 80%) between human and rat MBP sequences (Martenson, 1980) made it possible to purify human c-DNA sequences with a rat c-DNA probe. In the

first library screening step (Fig. 5), nearly 1/1000th (350/400,000) of the plaques were positive; this was probably due to the reduced stringency in hybridization conditions rather than to abundance of the MBP c-DNA's. Expression of MBP gene is low in adult brains and even in brain tumor cells, as in the library used here, the mRNA's coding for MBP are not abundant species. Several c-DNA inserts obtained after digestion of recombinant phage DNA with restriction endonuclease EcoRI contained MBP or MBP-like sequences; one of these MBP-positive c-DNA insert was approximately 1.3 kb and another one was 1.0 kb (Fig. 8). The 1.3 kb insert was gel-purified and the pattern of restriction endonuclease sites obtained following digestion with BamH1, PstI and PvuII (Fig. 8) strongly suggested it was the 1.3 Kb EcoRI c-DNA fragment that contained the human MBP coding region. The 1.0 kb DNA insert was not cleaved by BamH1, BglII, HincII, PstI or PvuII but contained 2 XbaI sites as expected for the non-coding, 1.0 kb EcoRI fragment of the human MBP c-DNA. Furthermore, hybridization of the 1.0 kb EcoRI DNA insert, to rat sequences was always weaker than the 1.3 kb DNA fragments. The coding region of human MBP c-DNA shows nearly 90% homology to the rat's and mouse's while the non-coding regions of the c-DNA's are only 65-70% homologous. Taking all these observations together, although these c-DNA inserts were not verified by sequencing, they represented authentic MBP sequences and together constituted the full length human MBP c-DNA.

CHAPTER 4

A POLYMORPHIC HUMAN MBP GENE

I. Introduction

Nucleotide base changes occur frequently in the human genome, on the average of one for every 200 to 300 base pairs. Because they do not always affect gene products and have a tendancy to cluster in the non-expressed regions of the genome (Peltonen and Palkkinen, 1986), these nucleotide base changes or polymorphisms are usually difficult to identify. As discussed in the Literature Review, many of the DNA polymorphisms involve insertions, deletions or rearrangements of sequences and create or destroy restriction enzyme sites. However, unless using the proper enzymes and gene probes, they can go undetected. In fact, RFLP's around many gene loci were found by chance, during the characterization of genomic DNA by restriction mapping or by sequencing.

This chapter reports the presence of DNA polymorphisms within and in the vicinity of the MBP gene in humans; these DNA polymorphisms were detected with several restriction enzymes, including PvuII and HincII, using a MBP c-DNA probe for DNA hybridization.

II. Results

A. EcoRI and BamH1 DNA polymorphisms in human MBP genes

In search for the MBP gene, southern blots similar to the blots employed in Fig. 4 were prepared. High molecular weight (MW) DNA purified from the same placenta as used in Fig. 4, was digested with restriction endonuclease EcoRI or BamH1. After transfer of the fragmented DNA to nitrocellulose paper, the blots were hybridized to the 1.5 kb c-DNA probe containing rat MBP sequences. The 1.5 kb c-DNA probe was radiolabeled by nick-translation. Several hybridization fragments were obtained with both EcoRI (Fig. 9, Lane 1) and BamH1 (Fig. 10, Lane 1); these included a 5.8-6.0 kb EcoRI fragment and a 4.0 kb BamH1 fragment which were probably the same fragments observed in Fig. 4 which hybridized to the mixed probe B. These 2 fragments contained sequences from exon 3 and exon 4 of the human MBP gene. To confirm the restriction patterns for EcoRI-or BamH1-digested placental DNA when probed with the rat MBP c-DNA probe, the hybridization experiment was repeated using DNA prepared from another placenta (DNA 6 in lane 6 of of figures 9 and 10). The same number of fragments, 6 for EcoRI digests and 7 for BamH1 digests were obtained. However, 3-4 of the higher MW fragments migrated slightly slower in the DNA prepared from the second batch of placenta (Fig. 9, Lane 6; Fig. 10, Lane 6) than in the first DNA preparation (Fig. 9, Lane 1; Fig. 10, Lane 1). Because of these differences in migration in

Fig. 9. Autoradiogram of placental DNA digested with restriction endonuclease EcoRI and hybridized with the rat MBP c-DNA probe. DNA was prepared from 8 different placentae. The bars indicate the 4 polymorphic fragments in lane 4 with their corresponding alleles in lanes 5 and 6. The strong hybridization bands of approximately 1.7 kb possibly reflect contamination with plasmid DNA. The sizes of the fragments are given in kilobases.

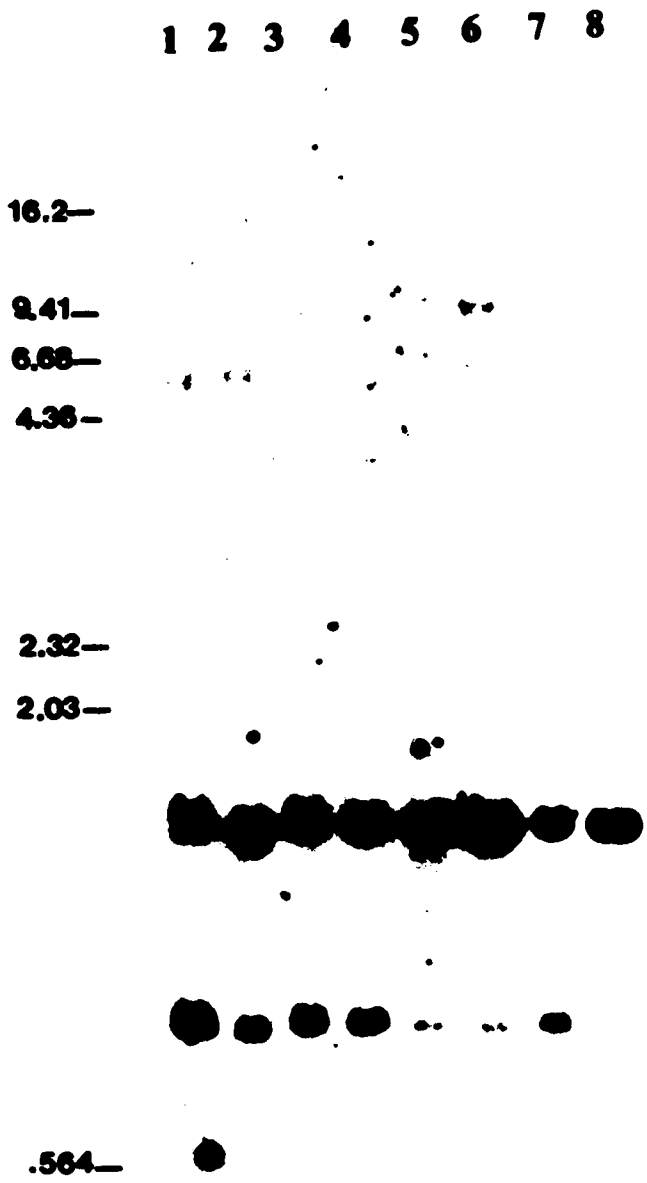
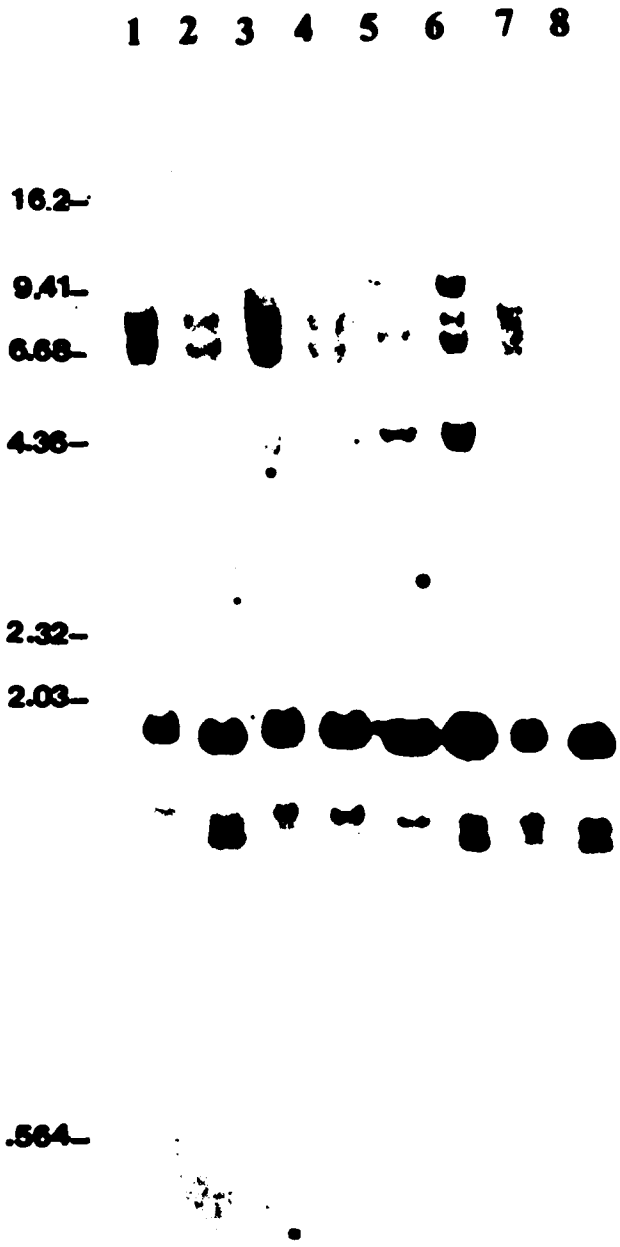

1 2 3 4 5 6 7 8
16.2—
9.41—
6.68—
4.36—
2.32—
2.03—
.564—

Fig. 10. Autoradiogram of placental DNA digested with restriction endonuclease BamH1 and hybridized with the rat MBP c-DNA probe. DNA was prepared from 8 different placentae. The strong hybridization bands of size 1.9 kb possibly reflect contamination with plasmid DNA. The sizes of the fragments are given in kilobases.


1 2 3 4 5 6 7 8
16.2
9.41
6.68
4.36
2.32
2.03
.564

some DNA bands, 6 additional placental samples were collected, their DNA extracted, digested with EcoRI or BamH1, blotted and hybridized under the same conditions as above. Fig. 9 and Fig. 10 compared the hybridization patterns of these 8 different placental DNA digested with EcoRI and BamH1 respectively. Lanes and lanes 6 in both Fig. 9 and Fig. 10 represented the patterns obtained with DNA from the first placenta (DNA1, same placenta as in Fig. 4) and from the second placenta (DNA6), respectively. There were two general hybridization patterns for each of the restriction enzyme digests. In the EcoRI digests, 6 out of 8 placental DNA (Fig. 9, lanes 1,2,3,4,7 and 8) showed MBP-hybridization bands of approximately 15.0 kb, 9.0 kb, 5.8 kb and 4.0 kb; in 2 out of 8 placental DNA (Fig. 9, lanes 5 and 6) the EcoRI hybridization bands were 15.3 kb, 9.3-9.4 kb, 6.1 kb and 4.3 kb. Similarly, with the BamH1 digests, 6 out of 8 placental DNA (Fig. 10, lanes 1,2,3,4,7 and 8) contained MBP sequences on DNA fragments with sizes of 9.8 kb, 7.5 kb, 6.6 kb and 4.0 kb while the other 2 placental DNA (Fig. 10, lanes 5 and 6) contained BamH1 bands of 10.2 kb, 7.8 kb, 6.9 kb and 4.3 kb. For each of the restriction enzyme digests, the differences in migration patterns involved several bands. This effect could result from variations in salt concentration in the different DNA samples. However, the experiments were repeated using different DNA preparations from each of the 8 placenta and the DNA samples from placenta 5 and from placenta 6 were always the only samples containing slower

migrating bands. Furthermore, some bands, for example the 0.9 kb EcoRI and the 1.68 kb- 1.62 kb BamHI, were present in all the 8 placental DNA.

Strong hybridization bands with size approximately 1.9 kb were observed in all the 8 placental DNA, with both the EcoRI and the BamHI digests. Although they did not hybridize with pBR322 sequences (data not shown), they possibly result from contamination with some plasmid or other small DNA.

Since the 6 placental DNA samples containing the faster migrating EcoRI bands also contained the fast migrating BamH1 bands, the differences in hybridization patterns observed with both enzymes must have resulted from a single mutation around the MBP gene which affected both enzyme sites. These mutations are likely to be insertions-deletions of 300 to 400 bp since the migration difference between corresponding alleles for each of these polymorphic fragments was in the 300-400 bp range. The 5.8 kb EcoRI fragment and the 4.0 kb BamH1 fragment, believed to include sequences from exon 3 and exon 4 of the human MBP gene, both were alleles in the insertion-deletion mutation; therefore, the EcoRI, BamH1 polymorphism reported here must be located near the third and fourth exons of the human MBP gene. The invariant 0.9 kb EcoRI band might be identical to the 0.9 kb EcoRI fragment shown by Boylan and coworkers (1986) to contain sequences from exon 1 of the human MBP gene.

B. Search for DNA polymorphism in the MBP gene with different restriction enzymes.

Two general hybridization patterns were obtained when DNA from 8 unrelated individuals (placentae) was digested with either EcoRI or BamH1; these 2 patterns are represented by placenta DNA1 (lane 1) and DNA6 (lane 6) in Fig. 9 and Fig. 10. In order to see whether other DNA polymorphisms could be detected around the MBP gene, samples of DNA1 and DNA6 were digested with several enzymes and the digests were hybridized to the 1.5 kb rat MBP c-DNA probe.

Fig. 11 compares the patterns obtained following digestions with restriction enzymes AvaII, BclI, BglI and BglII; the EcoRI and BamH1 digests were also included. Fig. 12 shows the hybridization patterns for digestion of DNA1 and DNA6 with restriction enzymes PvuI, PvuII and SalI. The hybridization band patterns from many of these enzyme digests were complex, with variations in hybridization intensity between bands of different MW, but differences between the 2 DNA samples (DNA1 and DNA6) could still be easily identified. The BamH1 digests in Fig. 11 showed more hybridization bands than expected (compared to Fig. 10) due to some incomplete digestion. There was no polymorphism between DNA1 and DNA6 for the AvaII, BglI or PvuI enzymes. However, migration differences similar to those observed in EcoRI and BamH1 restriction patterns were obtained in digests with BclI, BglII and PvuII. Again, placental DNA1 showed polymorphic

Fig. 11. Comparison of MBP-hybridization patterns of placental DNA1 and DNA6 when digested with restriction endonucleases AvaII, BamH1, Bcl1, BglI, BglII and EcoRI. The fragment sizes are given in kilobases.

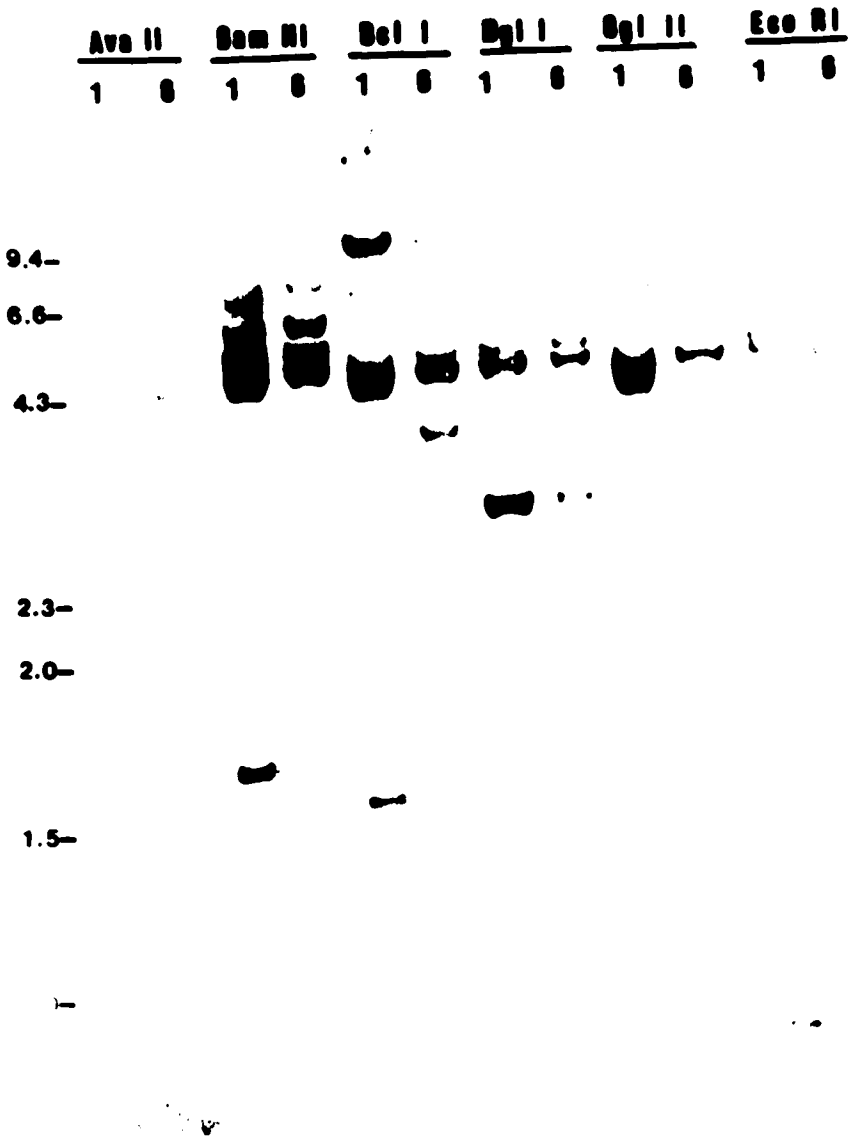
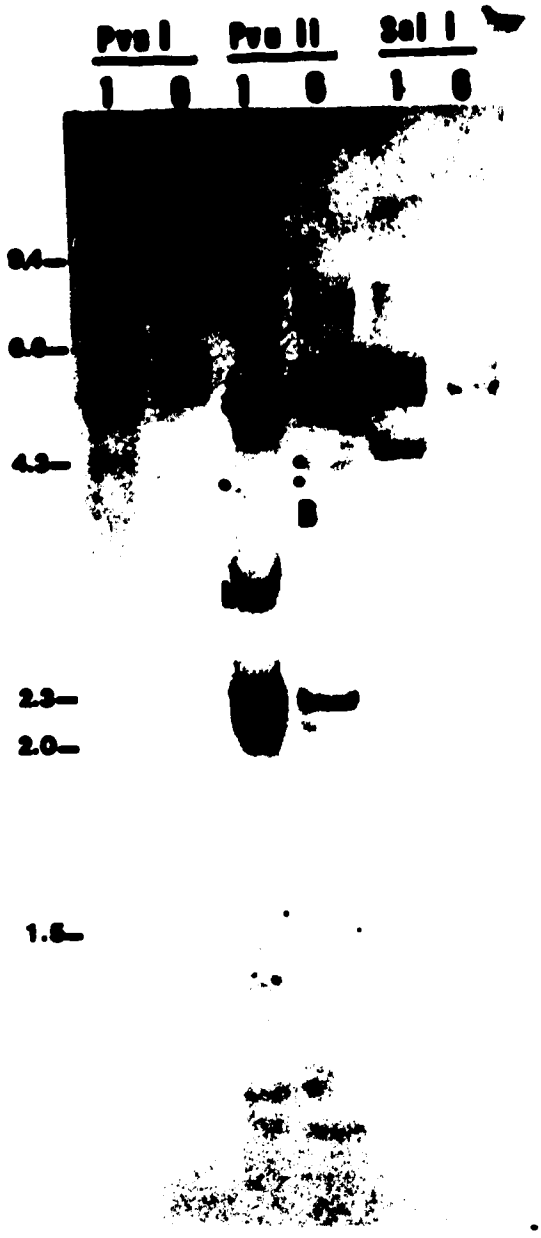

Ava II
Bam HI
Bcl I
Bgl I
Bgl II
Eco RI
1 6 1 6 1 6 1 6 1 6 1 6
9.4–
6.6–
4.3–
2.3–
2.0–
1.5–

Fig. 12. Comparison of MBP-hybridization patterns of placental DNA1 and DNA6 digested with the designated restriction enzymes. Slower migrating polymorphic fragments in DNA6 are indicated with capital letters, the corresponding alleles in DNA1 are indicated with the same lower case letter. Different letters are used for different polymorphisms. The 2 alleles of a polymorphism affecting a single fragment are indicated with a black dot in the PvuII lanes (these bands were more visible in the original blots; their presence was confirmed by a second observer). The fragment sizes are given in kilobases.


Pvu I
Pvu II
Sal I
1
0
1
0
1
0
9.4
6.6
4.3
2.3
2.0
1.5

bands migrating faster than their equivalents in DNA6. The lack of a size marker more precise than the λ HindIII and λ AvaI used here, made it difficult to give the exact size of the various bands with certainty, but the relative fragment size differences between DNA1 and DNA6 was still in the 300-400 bp range for most polymorphisms. For example, the BglII polymorphic fragments in DNA1 were 8.4 kb, 4.3 kb and 4.1 kb with the corresponding fragments in DNA6 being 8.8 kb, 4.7 kb and 4.4 kb, respectively.

At least two different types of DNA polymorphism (labeled A-a, B-b in Fig. 12) were detected with PvuII enzymes. The 300-400 bp insertion-deletion type involving EcoRI, BamH1 and BglII (polymorphism A-a in Fig. 12) were also observed in the PvuII band patterns. The polymorphic PvuII fragments were approximately 6.1 kb, 4.7 kb, 4.4 kb in placenta DNA1 and 6.5 kb, 5.1 kb and 4.8 kb in placenta DNA6. The other difference in hybridization patterns between DNA1 and DNA6 (polymorphism B-b, Fig. 12) was observed with PvuII bands of size 3.0-3.1 kb in placental DNA1 and 3.6 kb in placental DNA6. These bands were also detected by Boylan's group (1987) who suggested that these PvuII polymorphisms resulted from a single mutation.mapping in the 5' flanking region of the human MBP gene. A 3.8 kb PvuII band in DNA1 and the corresponding 4.3 kb band in DNA6 (labeled with black dots, Fig. 12) which may be different from the other two PvuII polymorphisms, were also detected recently by Kamholz

and coworkers (1987) during hybridization of human DNA to a c-DNA probe containing the entire human MBP coding region.

Except for the PvuII 4.3 kb-3.8 kb polymorphism where DNA1 is clearly homozygous for the 3.8 kb allele and DNA6 heterozygous, it was difficult to establish heterozygosity from these complex hybridization patterns where the hybridization intensity of some bands was very weak. For example, in SalI digests (Fig. 12), there is an additional 1.5 kb band in placenta DNA1, its corresponding allele could not be identified and placenta DNA1 may or may not be homozygous for this 1.5 kb SalI allele. Because this SalI polymorphism and the PvuII polymorphism involving a 3.0 kb allele and a 3.6 kb allele were probably different from the 300-400 bp insertion-deletion polymorphism of exon 3 - exon 4, they were studied in more detail.

C. Frequency of PvuII polymorphisms of the MBP gene in general population.

High MW DNA was prepared from 20 different placentae including the placentae 1 to 8 of Fig. 9 and Fig.10. The presence or absence of the 3.0 kb PvuII allele was in general more easily detected than the 3.6 kb allele. It was monitored after digestion with PvuII and hybridization with the rat c-DNA probe. The autoradiogram of Fig. 13 revealed 3.0 kb PvuII alleles for placental DNA1, DNA4, DNA5; this 3.0 kb PvuII band was also present in three additional DNA

samples (data not shown), making a total of 6 out of 20 placental DNA samples with the 3.0 kb PvuII allele, a frequency of 30.0% The 3.6 kb band could also be seen in DNA5 which is heterozygous. These blots were blindly read by a second observer who confirmed the presence of the designated hybridization bands. Placental DNA prepared here was mainly of maternal origin; however, because it could well be contaminated with fetal DNA, the frequency of polymorphism was also assayed for DNA prepared from peripheral blood. Blood was collected from 10 different individuals, 5 caucasians (Fig. 14, lanes B, D, F, G, H), 3 orientals (Fig. 14, lanes A, I, J) and 2 blacks (Fig. 14, lanes C, E). The 3.0 kb PvuII band was observed in DNA samples E, F and H only. None of the oriental's DNA samples showed a 3.0 kb PVUII band. Three out of 10 DNA samples from randomly collected blood showed the minor 3.0 kb PvuII polymorphic variant, a frequency identical to the one measured for placental DNA samples. Again in the autoradiogram Fig. 14, it was difficult to identify heterozygous DNA samples since the 3.6 kb PvuII allele could not always be visualized.

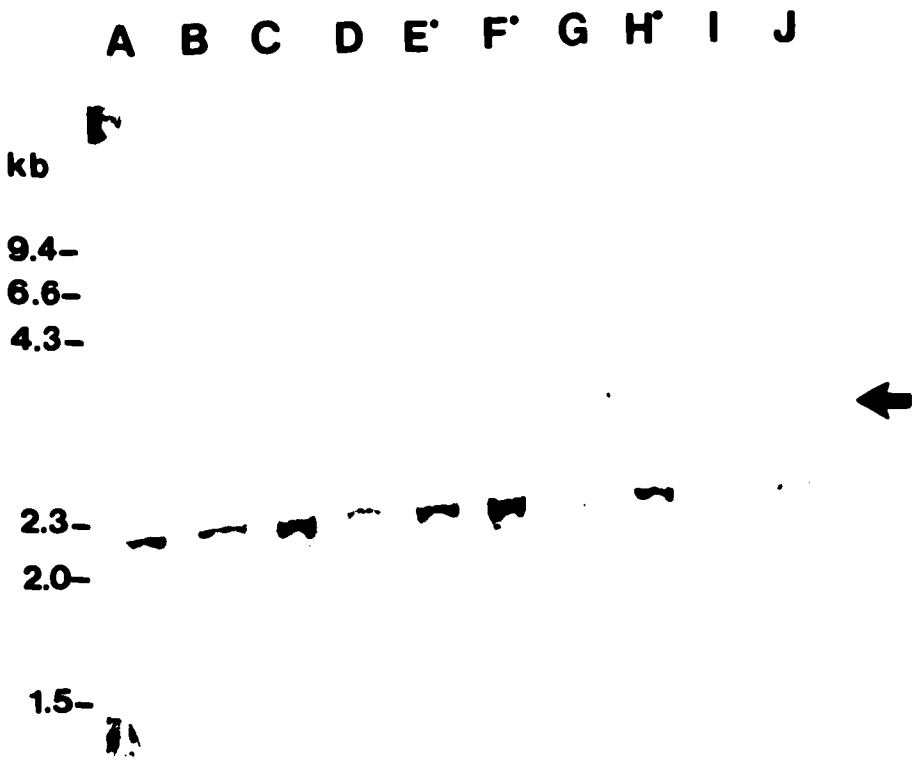
D. Frequency of HincII polymorphism of the MBP gene in the general population

SalI digestion of total human DNA followed by hybridization to a MBP c-DNA probe revealed a polymorphic 1.5 kb band (Fig. 12). SalI digestion, however, is sensitive to the state of methylation of the adenosine and cytosine

Fig. 13. MBP-hybridization patterns of 9 different placental DNA when digested with PvuII. The arrow marks the position of the polymorphic 3.0 kb variant present in some DNA samples (those marked with a black dot) and absent in others.


1
2
3
4
5
6
7
8
9
kb
14.6–
6.88–
4.71–
3.73–
1.88–
1.60–

Fig. 14. Hybridization patterns of 10 different DNA samples when digested with PvuII and hybridized with the rat MBP c-DNA probe. DNA was extracted from peripheral blood. The arrow indicates the position of the polymorphic bands. These polymorphic bands were more visible in the original blots; their presence was confirmed by a second observer. The samples containing the polymorphic 3.0 kb variant are indicated with a black dot.


A B C D E F G H I J
kb
9.4
6.6
4.3
2.3
2.0
1.5

residues in the recognition site GTCGAC; the SalI isoschizomer HincII (recognition site GTPyPuAC) which is insensitive to the methylation state was, therefore, tested. DNA polymorphism in the human MBP gene was also detected with the enzyme HincII, mainly as a 1.0 kb band found in the DNA samples that showed the 1.55 SalI band (data not shown). When the presence of a 1.0 kb HincII fragment was monitored among the 20 different placental DNA (Fig. 15), 5 out of 20 samples (DNA samples 1, 4, 5 in Fig. 15) *i.e.* 25%, showed the polymorphic variant; these DNA samples also contained the 3 kb PvuII polymorphic allele of the MBP gene. The 1.0 kb band was very faint and difficult to visualize in DNA sample 1 where the 3.0 kb PvuII was easily recognized.(Fig. 13).

Among the 10 peripheral blood DNA samples (Fig. 16), DNA samples E, F and H that were positive for the 3.0 kb PvuII allele also showed the 1.0 kb HincII band; the frequency of 1.0 kb HincII polymorphism was 30.0% among blood DNA samples from unrelated individuals.

III. Discussion

Screening DNA from unrelated individuals with a c-DNA probe encoding the small rat MBP, revealed the existence of several polymorphic sites within or near the human MBP gene. There are indications that at least some of these polymorphic sites, including the EcoRI and the BamH1 sites, must reside in the central portion of the gene, near the third and fourth

Fig. 15. MBP-hybridization patterns of HincII-digested placental DNA samples. DNA was prepared from 9 different placentae. An arrow marks the position of the variant 1.0 kb fragment. The black dots indicate DNA samples with the polymorphic variant. The bands observed in DNA sample 7 were not reproducible and were not considered as genuine polymorphic bands.

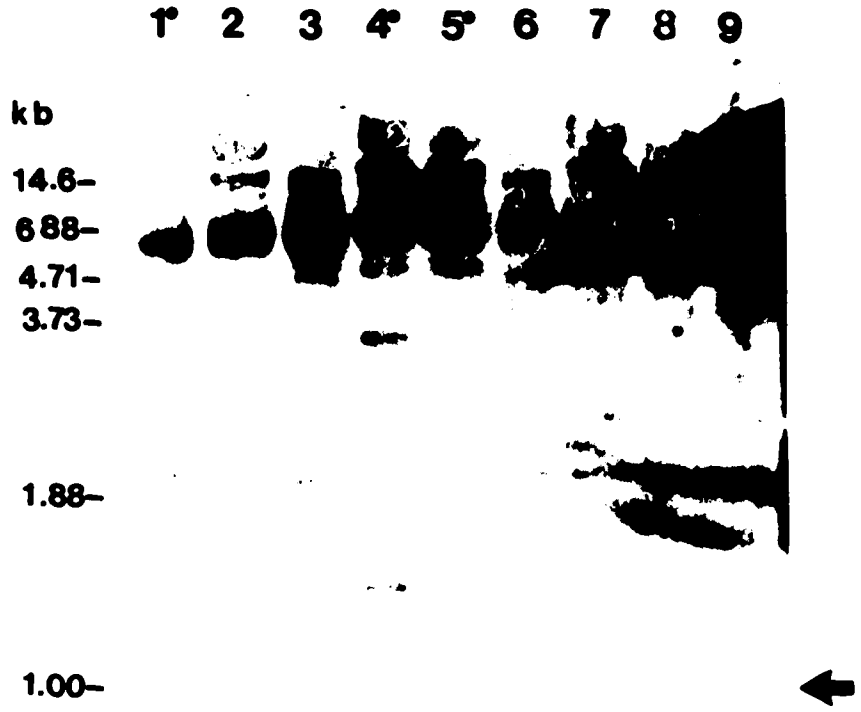
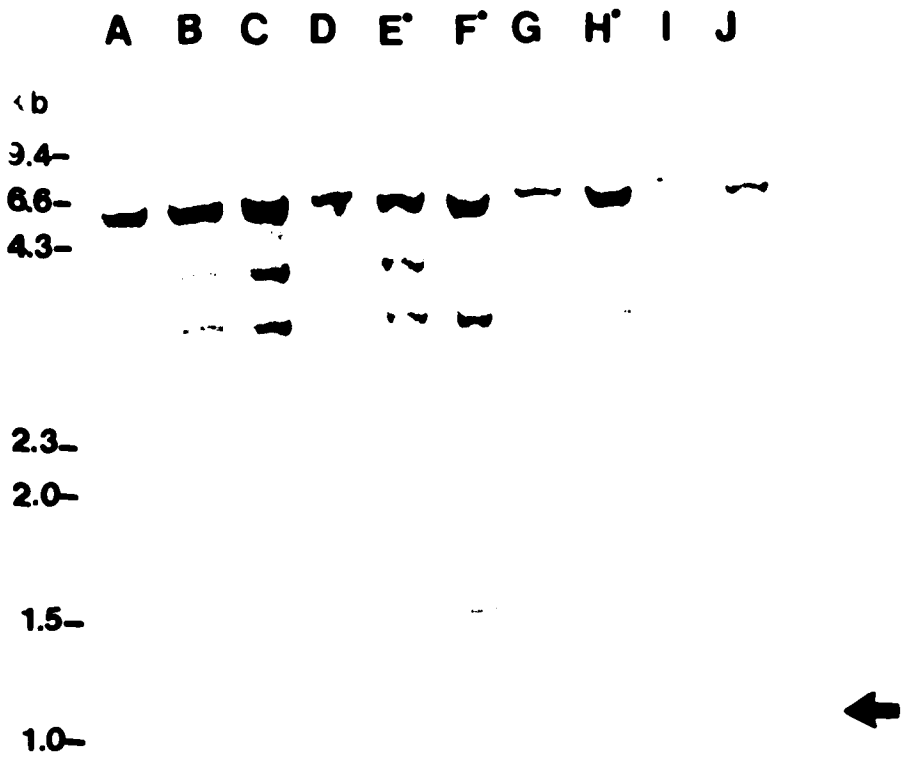

1
2
3
4
5
6
7
8
9
kb
14.6–
6.88–
4.71–
3.73–
1.88–
1.00–

Fig. 16. Hybridization patterns of 10 different DNA samples digested with HincII and hybridized with the rat MBP c-DNA probe. DNA was extracted from peripheral blood. The arrow indicates the position of the polymorphic 1.0 kb fragment. The black dots indicate DNA samples with the polymorphic fragment.


A B C D E F G H I J
kb
9.4
6.6
4.3
2.3
2.0
1.5
1.0

exon. First, for both of these enzymes, one of the variant fragments, the 5.8 kb EcoRI and the 4.0 kb BamHl, was also detected using the mixed synthetic probe B (Fig. 4, Chapter 3). Probe B was derived from the reverse translation of amino acids 87 to 96 of the human MBP (Fig. 2, Chapter 3), a peptide that is encoded by exon 3 and partly by exon 4 according to the human MBP gene organization suggested by Roth and coworkers (1987). Secondly, the rat c-DNA used as probe in these studies was lacking sequences corresponding to exon 2 and exon 6 of the human gene; therefore, due to the size of the gene and the presence of very large introns, the EcoRI and BamHl polymorphisms will likely not reside within exon 2 and exon 6. Thirdly, recently, 2 groups have independently reported several polymorphic changes around the human MBP gene (Boylan *et al.*, 1987; Kamholz *et al.*, 1987). Using a MBP exon-1 probe, one of these groups failed to detect any EcoRI polymorphism in exon 1 or in the 5' MBP flanking region. The other polymorphic sites reported by that group, for example the PvuII 3.6 K-3.1 kb alleles, were also observed, among other polymorphisms, in the present study. The EcoRI polymorphisms detected with the rat c-DNA probe is, therefore, not found within or near exon 1.

The hybridization patterns presented in Fig. 11 and Fig. 12 agreed very well with the patterns shown by Boylan's group (Boylan *et al.*, 1987) for PvuII and by Kamholz's group (Kamholz *et al.*, 1987) for BamHl and PvuII; these groups have

used human MBP exon 1 probe and human c-DNA probe respectively. As expected,the PvuII fragments (3.6 kb, 3.1 kb) detected using the exon 1 probe were also revealed by the rat c-DNA probe (Fig. 12). Similarly, many of the BamH1 and PvuII fragments hybridizing to the human c-DNA probe also hybridized to the rat c-DNA probe. The human MBP c-DNA probe used by Kamholz's group revealed BamH1 fragments of sizes 10.8 kb, 6.7 kb, 5.7 kb, 5.5 kb and 3.5 kb with the 5.7 kb and 5.5 kb as variant fragments; fragments smaller than 3.5 Kb were not shown in their blots. The rat c-DNA probe in Fig. 11 revealed BamH1 fragments that were mainly polymorphic but with sizes equivalent to some of the fragments detected with the human c-DNA probe, *i.e.* 10.2 kb or 9.8 kb, 7.8 kb or 7.5 kb, 6.9 kb or 6.6 kb, 4.3 kb or 4.0 kb alternative alleles. The minor differences between band patterns following hybridization to human c-DNA and those in Fig. 11 might result from the possible presence of one or two additional exons (exon 2 and/or exon 6) in the human c-DNA probe and most likely from the difficulty to assign exact size to the higher MW bands under the present electrophoretic conditions (0.8 % agarose gels, λ HindIII size-markers). PvuII fragments detected with the human c-DNA probe were the allelic 4.3 kb and 3.8 kb, 3.6 kb and 3.1 kb, 2.5 kb, 2.3 kb, 1.7 kb, 1.3 kb; the bands with MW greater than 4.3 kb were not shown. The equivalent PvuII bands in Fig. 12 had sizes of 4.3 kb and 3.8 kb, 3.6 kb and 3.0 kb, 2.4 kb, 2.3 kb, 1.5 kb, 1.1 kb.

In summary, DNA polymorphic changes affecting the human MBP gene have been detected with several enzymes; these included the restriction enzymes BamH1, BclI, BglII, EcoRI, PvuII and SalI or HincII. The enzymes AvaII, BglI and PvuI failed to reveal any polymorphism in the present system. Although the possibility of differences in migration resulting from differences in salt concentrations was not completely ruled out, the polymorphisms detected with the above enzymes probably result from a single mutation. For each of these enzymes, the same number of polymorphic fragments (3 or 4) were observed; and, when comparing the 2 placental DNA tested (DNA1 and DNA6), these polymorphic bands differed in size relative to one another in a pattern common to all the enzymes. These data suggested that the mutation responsible for some of the observed polymorphisms could not be the loss or the gain of a restriction site but rather a mutation involving DNA insertions, deletions or both. Because the relative fragment size differences of the polymorphic bands between the two different DNA samples were 300 to 400 bp, the mutation must involve insertion-deletion of 300 or 400 bp. Length polymorphisms similar to the ones described here (which affected EcoR^{I}, BamH1, BglII, HindII, KpnI, PstI and PvuII) seem to be common in human genes, where they appear frequently in the 5' flanking regions. For example, polymorphisms occur in the 5' flanking regions of the insulin gene (Bell *et al.*, 1982), the c-Ha-ras-1 oncogene

(Capon *et al.*, 1983), the α-globin gene cluster (Proudfoot *et al.*, 1982; Goodbourn *et al.*, 1983) and more recently the human MBP gene (Boylan *et al.*, 1987). In all these cases, the basis for the length polymorphisms is the presence of tandem repeats of short sequences or minisatellites (Jeffreys *et al.*, 1985) where variations in the number of repeats cause the polymorphism. Intragenic length polymorphisms are not as well documented, the best studied case being the myoglobin gene which contains 4 tandem repeats of one 33-bp sequence in the 3' end of intron 1 (Weller *et al.*, 1984); the human zeta-globin gene has a 14-bp tandem repeat [illegible] in the first intron (Proudfoot *et al.*, 1982). The data presented here do not allow the precise mapping of the EcoRI-BamHl-polymorphism within the human MBP gene. However, the mutation is likely to be within introns rather than in the expressed sequences because an insertion-deletion 300 bp long within an exon would have profound consequences on the protein structure. Whether the 300 bp insertion-deletion is due to tandem repeats of mini-satellites cannot be determined either since DNA sequences of introns 2 and 3 of human MBP genes of placental DNA1 and DNA6 was not available. It is interesting to note that the insertion-deletion within the MBP gene has a size range common to many random-repetitive sequences dispersed in the human genome. The Alu family of repetitive sequences is 300 bp (Houck *et al.*, 1979), the EcoRI family is 340 bp (171 bp plus 169 bp) (Wu and Manuelidis, 1980) and the Hinf family repeat is 319 bp (Shimizu *et al.*, 1983).

The polymorphism involving PvuII fragments 3.6 kb and 3.0 kb was different from the insertion-deletion polymorphism with EcoRI-BamH1 enzymes. Placental DNA5 and DNA6 in Fig. 9 and Fig.10 showed the same EcoRI and BamH1 patterns, with slower migrating polymorphic bands. Fig. 13a showed that placental DNA5 contained the 3.0 kb PvuII allele while DNA6 did not. The 3.0 kb PvuII polymorphism may also be of the insertion-deletion type since all the placental DNA (Fig. 13a, Fig. 13b) and peripheral blood DNA (Fig. 14) which showed a 3.0 kb PvuII band, also showed a 1.0 kb HincII fragment (Fig. 15a, Fig. 15b, Fig. 16). Boylan and coworkers (1987) reported a PvuII polymorphism involving two bands of approximately 3.5 kb and 3.6 kb plus a third band of 3.1 kb; this polymorphism was part of a length variation mutation found in the 5' end of the human MBP gene and which affected many other enzymes. PvuII polymorphism (3.6 kb-3.0 kb alleles), and possibly HincII polymorphism (1.0 kb band) resulted from a single mutation; this mutation might be identical to the length variation found in the 5' flanking region, 0.5 kb to 2 kb upstream of the human MBP gene (Boylan *et al.*, 1987).

CHAPTER 5

DNA POLYMORPHISMS OF MBP GENE IN PATIENTS WITH MULTIPLE SCLEROSIS

I. Introduction

Multiple sclerosis (MS) is the most common demyelinating disease of the CNS in humans. Demyelinations in animals, such as the Jimpy mutation (Ikenaka *et al.*, 1988), the Quaking disease (Fujita *et al.*, 1988) or the Shiverer disease (Inoue *et al.*, 1988) are usually caused by absence of or defects in one of the structural component of myelin. The causes for demyelination in MS are not known but several factors are involved. There seems to be an initial infection with some slow virus which later, in some individuals, triggers an autoimmune reaction against one or more of the myelin components. MS is not an inherited disease but there are strong indications that genetic factors do influence disease risk (reviewed by McFarland *et al.*, 1984). First, MS is often a family disease affecting several members in some families. Secondly, twin studies show that chances of developing MS in both members of a twin pair are greater in monozygotic twins than in dizygotic twins. Thirdly, certain racial groups (Bantu, Maori, Hungarian Gypsies) are almost completely protected against MS. Genes of the major histocompatibility region, HLA-DR region (reviewed by Tiwari and Terasaki, 1985) and the immunoglobulin structural gene region, particularly immunoglobulin G1 (Haile *et al.*, 1985) and G3 (Gaiser *et al.*, 1987) have been found associated with

susceptibility to MS in many cases. HLA-DR and immunoglobulin G genes are known to control immune responses to a wide variety of antigens and are associated with susceptibility to many diseases that show, like MS, some autoimmune component (Pollack *et al.*, 1983; Willcox *et al.*, 1985). However, these 2 gene complexes alone cannot explain the whole MS spectrum and other genes are likely to be involved. The genes coding for the myelin components PLP, MAG and especially MBP constitute perfect candidates in search of an association with the development of MS. MBP is considered an important, if not the major antigen in the autoimmune attack of myelin in MS (Warren and Catz, 1987). The MBP isolated from MS brain is, however, not different in physical and immunogenic properties from the protein in normal brains. Preliminary studies compa g charge microheterogeneity, amino acid composition and partial amino acid sequences, show no obvious differences between the MBP from the brains of MS patients and that from control brains (Chou *et al.*, 1978; Wood and Moscarello, 1984). The only change reported so far has been a conservative amino acid change, with the substitution of a serine for a glycine residue in the protein (between residues 44 to 49) from one MS patient. Whether silent mutations of this sort affecting the protein itself and not its immunogenic properties or silent mutations in the non-expressed regions of the MBP gene are factors protecting or predisposing to MS is not known. Results in Chapter 4 showed many DNA polymorphic changes

within and around the human MBP gene; some of these polymorphisms, detected with PvuII, PstI, HincII and SalI, which lie in the 5' flanking region did not affect the primary structure of the gene product but may alter overall gene expression. It is interesting to note that in the myelin-deficient mice, an insertion or rearrangement interrupting the 5' regulatory region in only one of 2 copies of the MBP gene is the mutation responsible for the lack of organized myelin and the subsequent neurological problems, tonic convulsions and ataxic movements (Akowitz *et al.*, 1987).

This chapter assesses frequency of PvuII and HincII polymorphisms of the MBP gene in MS patients as compared to race, sex and age-matched controls. The MS patients analyzed (ages ranging from 22 to 60 years) were all of the chronic MS type with some in which the neurological problems progressively worsen (active progressive MS), some in which the MS shows exacerbations and relapses (active relapsing MS) and a few patients in which MS was diagnosed but no attacks had occured in 10 or more years (inactive MS patients).

II. Results

A. PvuII-HincII polymorphisms of the MBP gene in MS patients

Peripheral blood was collected from 34 MS patients and 34 matched controls selected through the MS clinic. The samples were coded 1 to 68 and the entire DNA analysis was performed blindly, without any information regarding the medical history of the donors, simply using the code number as provided by the MS clinic. DNA was extracted immediately after blood collection and the purified DNA samples were digested with either PvuII (Fig. 17a and 17b) or HincII (Fig. 18) before blotting and hybridization to the c-DNA probe containing rat MBP sequences. PvuII polymorphisms involved 2 alleles (3.6 kb and 3.1-3.0 kb). However, because the 3.6 kb band was often more difficult to visualize, PvuII polymorphisms in the general population (controls) was defined by either the presence (minor polymorphisms) or the absence of the 3.0 kb hybridization band. Similarly, the HincII polymorphism was defined here by the presence or absence of a 1.0 kb HincII band. The results obtained in the DNA analysis of the 68 patients (Table II; Fig. 17a, 17b and 18) were blindly read and, with the exception of two samples, confirmed by two different observers. The DNA samples from patients 6 and 51 showed faint signals for both the 3.0 kb PvuII and the 1.0 kb HincII bands; it was therefore difficult to draw any conclusion. However, in Table II, DNA sample 6

is considered positive (asterisk sign) and DNA sample 51 negative, based on the decision of two out of three observers. This decision should not affect the statistical analysis to a great extent since patient 6 had MS and patient 51 did not.

Fig. 17a and Fig. 17b show examples of the patterns obtained for PvuII digests of DNA samples from 20 of the 68 blood donors of the MS clinic. A 3.0 kb band was observed for sample 25 in Fig. 17a and samples 64 and 65 in Fig. 17b. The hybridization bands in samples 25 and 64 were very faint but still visible in the original blots; unfortunately reproduction from the original blots decreased the intensity of the bands. This problem was also encountered with other DNA samples (data not shown). Samples 61 and 65 also contained the alternative 3.6 Kb PvuII allele. DNA samples in which neither the 3.0 Kb, nor the 3.6 Kb PvuII bands could be detected,as in Fig. 17b (sample 62) or Fig. 17a (sample 26) were often re-examined and occasionally showed weak 3.6 kb bands, but never any bands corresponding to the 3.0 kb fragment (data not shown). Among the 68 peripheral blood DNA samples obtained from MS clinic and listed in Table 21 contained the 3.0 kb PvuII bands, *i.e.* DNA samples 8, 9, 11, 14, 16, 17, 25, 28, 31, 34, 38, 44, 45, 5[illegible] 6[illegible] 64 and 65.

Fig. 17-a. Autoradiogram of PvuII-digested DNA (isolated from patients 21 to 26 of the MS clinic) hybridized with a rat MBP c-DNA probe. The position of the polymorphic fragment is indicated by an arrow and the DNA sample containing the polymorphic 3.0 kb variant is marked with an asterisk.

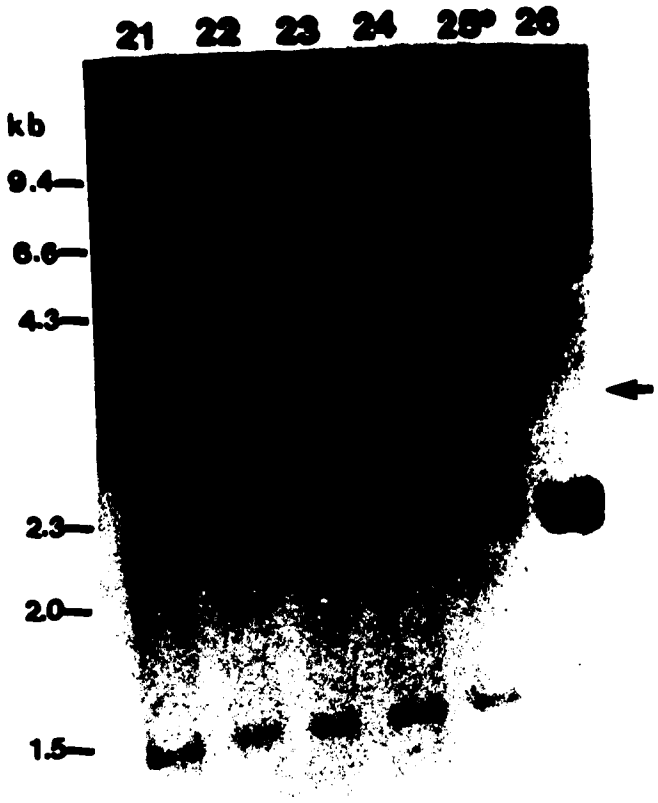

21
22
23
24
25
26
kb
9.4
6.6
4.3
2.3
2.0
1.5

Fig. 17-b. Autoradiograms of PvuII-digested DNA (from patients 55 to 68 of the MS clinic) hybridized with a rat MBP c-DNA probe. The position of the polymorphic 3.0 kb PvuII band is indicated with an arrow and DNA samples containing the polymorphic band are marked with an asterisk.

55 56 57 58 59 60 61 62 63 64 65 66 67 68
kb
94
66
43
23
20
14

Table II. Disease status of the 68 blood donors from the MS clinic.

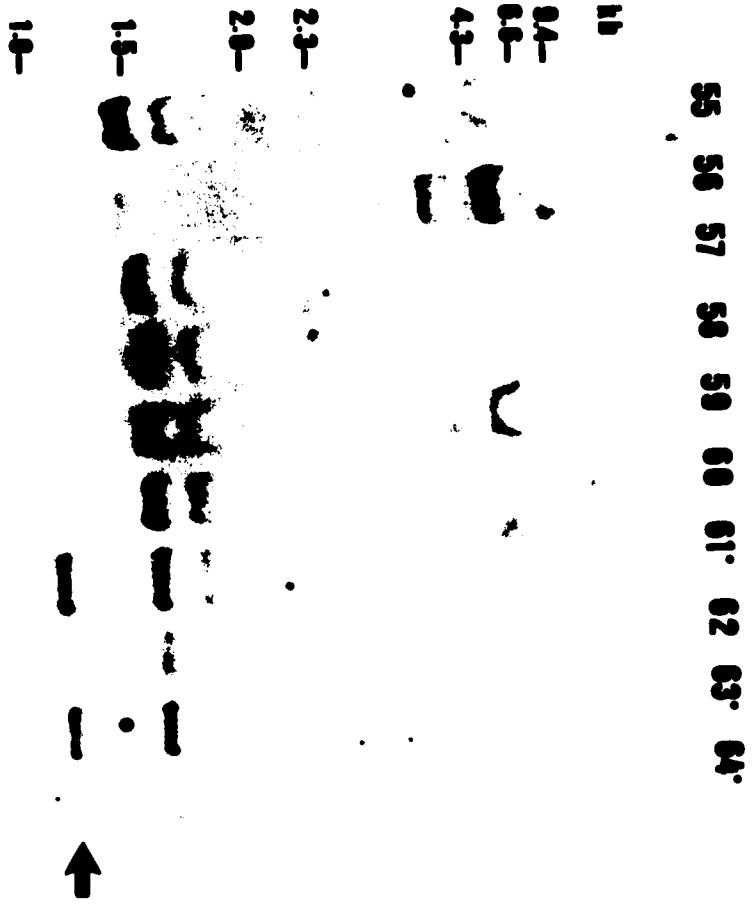
Subject (code number)	Disease status	Subject	Disease status	Subject	Disease status
1*	MS	24	MS	47	MS
2	MS	25*	MS	48	--
3	--	26	--	49	MS
4*	MS	27	MS	50*	MS
5	--	28*	MS	51	--
6*	MS	29	MS	52	--
7	--	30	MS	53	--
8*	--	31*	MS	54	MS
9*	--	32	MS	55	--
10	MS	33	MS	56	MS
11*	MS	34*	--	57	--
12	MS	35	MS	58	-
13	MS	36	--	59	--, father of 60
14*	--	37	--	60	MS
15	MS	38*	MS	61*	--
16*	MS	39	--	62	--, mother of 60
17*	--	40	MS		
18	MS	41	--	64*	--
19	MS	42	--	65*	--, father of 11
20	MS	43	--	66	--, mother of 11
21	--	44*	MS		
22	MS	45*	MS	67	--, mother of 32
23	--	46	--	68	--, father of 32

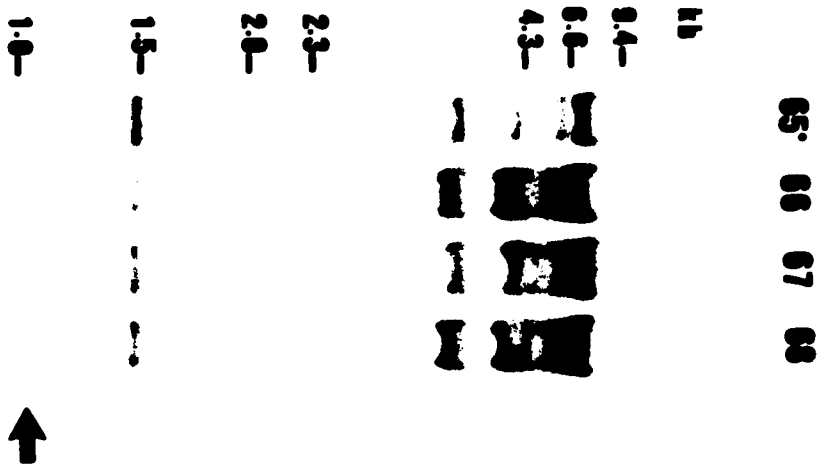
* denotes presence of the 3 kb PvuII - 1.0 kb HincII polymorphic bands in hybridization patterns fig. 17 and fig. 18.

Fig. 18 shows examples of HincII patterns for DNA samples 55 to 68 following hybridization to the rat MBP c-DNA probe. The HincII pattern was the same for all DNA samples except for the presence of a 1.0 kb band, the polymorphic variant, in some samples. Those samples are marked with a black dot in Fig. 18. The 1.0 kb bands appeared clearly in general except for samples 65 which was not as completely digested. When this sample was digested to completion, the HincII polymorphic variants were better visualized (data not shown). Again, among the 68 blood DNA samples analyzed, 21 showed the rare 1.0 kb HincII DNA polymorphism. These 21 samples, listed in Table II, were also the samples which showed a 3.0 kb PvuII allele These observations confirmed that presence of the 3.0 kb PvuII band correlated with the presence of a 1.0 kb HincII band. It, therefore, suggested that a single mutation, possibly insertion-deletion or rearrangement, was responsible for the DNA changes detected by these two enzymes.

To determine whether presence of the 3.0 kb PvuII and 1.0 kb HincII alleles could be associated with MS or not, the blood DNA samples were decoded and the disease status for each of the 68 blood donors examined. Table II shows the disease status for the 34 MS patients and the 34 healthy controls; the samples marked with an asterisk are those containing the 3.0 kb PvuII and 1.0 kb HincII bands. Among the 34 controls were the healthy parents of three of the MS

Fig. 18. Autoradiograms of HincII-digested DNA (from patients 55 to 68 of the MS clinic) hybridized with a rat MBP c-DNA probe. The positions of the polymorphic fragment are indicated by arrows and the DNA samples positive for the polymorphic 1.0 kb fragment are indicated with a black dot.





patients; unfortunately siblings and other family members for these three families were not available for pedigree analyses. However, despite the limited family information, the results in Table II indicated that at least for the three families available (families of patients 11, 32 and 60), the PvuII and HincII alleles segregated in a Mendelian fashion (Fig. 19). The 3.0 kb PvuII-1.0 kb HincII polymorphic variants of patient 11 must have been inherited from the father, *i.e.* blood donor 65, since the mother, blood donor 66, did not contain the polymorphic variants. Both MS patients 32 and 60 did not show 3.0 kb PvuII or 1.0 kb HincII bands and none of their parents, blood donors 67 and 68 and blood donors 59 and 62, respectively, was positive for these polymorphic variants.

With respect to frequencies of PvuII-HincII polymorphisms, Table II showed the minor polymorphic variants present in 12 out of 34 MS patients and in 9 out of 34 non-MS controls; this gave frequencies of 35.3% and 26.5% among MS patients and among controls, respectively. PvuII-HincII polymorphism frequencies measured in Chapter 4 in DNA from 20 placentae or from 10 randomly collected blood samples, were 30.0%. Medical histories for these DNA donors were not available. However, since none of them were known to show any sign of neurological disorder, they were also considered as controls, *i.e.* non-MS DNA donors. As indicated in Table III, the polymorphism frequency in MS patients (35.3%) was not significantly different from the frequency in controls,

Fig. 19. Inheritance of 3.0 kb PvuII - 1.0 kb HincII variant alleles in a family with one MS child. Siblings and other family members were not available for pedigree analysis.

The numbers (65, 66, 11) correspond to the code number as given by the MS clinic for the patients with disease status listed in Table II.

"+" denotes presence of 3.0 kb PvuII - 1.0 kb HincII alleles.

"-" denotes absence of 3.0 kb PvuII - 1.0 kb HincII alleles.

male

female

MS patient (female)

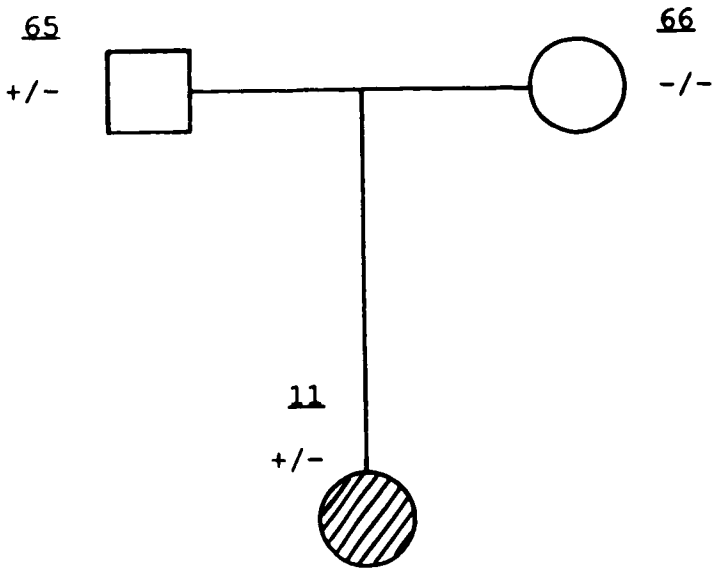

65
+/-
66
-/-
11
+/-

Table III. Presence of 3.0 kb PvuII - 1.0 kb HincII restriction fragments in DNA from controls and MS patients.

Study Number	Samples	Number of positive samples/Total number of samples	Percentage
Study 1	MS patients	12/34 [1,2]	35.3
	MS-matched controls	9/34 [1]	26.5
Study 2	Placenta	6/20	30.0
	Blood	3/10	30.0
Combined	Controls from Study 1 and Study 2	18/64 [2]	28.1

[1] Chi square, $\chi^2 = 0.689 < \chi^2_{.05} = 3.841$, degree of freedom, df = 1 for MS patients vs controls, Study 1.

[2] $\chi^2 = 0.555 < \chi^2_{.05} = 3.841$, df = 1 for MS patients vs combined controls.

[1,2] Proportion test values, Z = 0.7875 and Z = 0.7321 for MS patients vs controls, Study 1 and MS patients vs combined controls respectively; $Z_{.05} = 1.645$.

Relative incidence or risk factor,

[1] $\frac{12 \times 25}{9 \times 22} = 1.51$, MS patients vs controls, Study 1

[2] $\frac{12 \times 46}{18 \times 22} = 1.39$, MS patients vs combined controls

whether including control placental and randomly collected blood samples (28.1%) or just considering the 34 matched controls provided along with the MS patients (26.5%). At the 95% confidence level, there was no association between the presence of 3.0 kb PvuII or 1.0 kb HincII polymorphic variants and susceptibility to MS. The risk factor or relative incidence measured was only in the 1.4 to 1.5 range, not significantly greater than unity.

Among the 12 MS patients positive for the 3.0 kb PvuII-1.0 kb HincII alleles, 6 were females and 6 were males (Table IV); this suggested no correlation between this polymorphism and the sex of the MS patients: the chance of developing MS was not greater for a female with the polymorphisms than they were for a male with the same polymorphisms.

B. Relationship between PvuII-HincII polymorphism of the MBP gene and MS type

All of the 34 MS patients analyzed had the chronic type of MS. Six of them, the inactive MS patients, had had no neurological attack in the last 10 years; 8 patients showed a relapsing-remitting type of MS, with two or more attacks per year and the 20 remaining patients had progressively more severe neurological problems (Table IV). The frequencies of PvuII-HincII polymorphisms for the different MS types are compared in Table V. Fifty per cent of the inactive MS patients were positive for the rare variants as opposed to

Table IV. Disease features of the 34 MS patients tested for polymorphisms in the MBP gene.

Subjects	Disease Classification	Age of Disease Onset (years)	Sex
(Positive for 3.0 kb PvuII - 1.0 kb HincII alleles)			
1	Inactive	27	F
4	Progressive	32	M
6	Progressive	25	M
11	Relapsing	24	F
16	Progressive	24	F
25	Relapsing	19	F
28	Inactive	49	F
31	Progressive	42	M
38	Progressive	13	M
44	Progressive	27	F
45	Inactive	27	M
50	Progressive	22	M
(3.0 kb PvuII - 1.0 kb HincII alleles, absent)			
2	Inactive	32	M
10	Progressive	25	M
12	Relapsing	18	M
13	Inactive	27	M
15	Progressive	30	F

TABLE IV. Cont'd...

Subjects	Disease Classification	Age of Disease Onset (years)	Sex
18	Progressive	40	F
19	Progressive	19	F
20	Progressive	37	M
22	Progressive	25	F
24	Relapsing	37	M
27	Progressive	24	M
29	Relapsing	38	F
30	Relapsing	21	M
32	Progressive	29	F
33	Progressive	11	M
35	Relapsing	23	M
40	Progressive	43	F
47	Progressive	16	F
49	Progressive	29	M
52	Progressive	9	F
56	Inactive	36	F
60	Relapsing	17	F

Table V. Frequency of the rare[1] PvuII-HincII polymorphisms of the MBP gene in patients of different MS groups[2].

MS classification	Number of positive samples/Total number of samples	Percentage
Inactive	3/6	50.0
Active Progressive	7/20	35.0
Active Relapsing	2/8	25.0
Controls, non-MS	9/34	26.5

[1] Rare PvuII-HincII polymorphisms refer to presence of a 3.0 kb PvuII and a 1.0 kb HincII fragment.

[2] MS classification is according to Schumacher's criteria (Schumacher *et al.*, 1965) and is explained in text.

Chi-square, $\chi^2 = 1.8771 < \chi^2_{.05} = 7.815$, df = 3 comparing frequencies in controls and in each MS group.

$\chi^2 = 0.9473 < \chi^2_{.05} = 5.991$, df = 2 comparing frequencies among individual MS groups.

35% for patients with progressive MS and 25% for patients with relapsing-remitting MS. Chi-square probability tests performed to determine the significance of differences in these polymorphism frequencies, all indicated an absence of correlation between presence of the rare polymorphic variants and disease pattern among MS patients. The number of patients analyzed for some types of MS was small (only 6 patients with inactive MS) but probably sufficient to have indicated a strong association or at least a tendency (negative or positive) where present.

C. Relationship between PvuII-HincII polymorphism in the MBP gene and age of disease onset in MS patients

Besides the pattern of neurological attacks, one of the many variable parameters in the clinical course of MS is the age at which the first symptoms appear. In most cases of chronic MS, the age of onset is between 20 and 40 years, however, the disease is sometimes apparent in children or in teenagers, as for patients 38 and 52 (Table IV). In order to establish whether early or late disease onset could be influenced by one of the polymorphic variants analyzed here, the frequencies of polymorphism were measured in 3 groups with different ages of MS onset (Table VI). Of the 14 MS patients who developed disease before they were 25 years old, 5, *i.e.* 35.7%, showed the minor 3.0 kb PvuII-1.0 kb HincII variants. Similarly, only 5 out of the 17 MS patients with disease onset between age 25 and age 40 showed the minor polymorphic variants, a proportion of 29.4% which is not

Table VI. Relationship between age of MS onset and frequency of PvuII-HincII polymorphisms of the MBP gene.

Age of onset (years)	Number of positive[1] samples/Total number of samples	Percentage
below 25	5/14	35.7
25 - 40	5/17	29.4
above 40	2/3	--

[1] Refers to the presence of both the 3.0 kb PvuII and 1.0 kb HincII fragments.

statistically different from the 35.7% observed for the MS group with age of onset before 25. Only 3 patients developed MS after 40 years of age, 2 showed the minor polymorphic variants. However, the numbers for the late disease onset group of MS patients are probably too small to be conclusive.

There is, therefore, no clear association between PvuII-HincII polymorphism in the 5' flanking region of the MBP gene and age of MS onset; the frequency of polymorphism in these MS groups with different ages of disease onset were in fact not different from the frequencies observed in control non-MS patients (28-30%).

II. Discussion

Sixty eight different peripheral blood DNA samples were digested with PvuII or HincII and the DNA digests hybridized to a MBP probe in search for a 3.0 kb PvuII band and a 1.0 kb HincII band which are alleles of a polymorphism mapping in the 5' flanking region of the human MBP gene. In many of the blots similar to those shown in Fig. 17 and Fig. 18, several hybridization bands mainly those with high MW, were faint and even the 3.0 kb PvuII band was sometimes difficult to visualize. These differences in hybridization intensity might result from factors such as a) low efficiency of transfer of some bands (despite the acid treatment), b) hybridization of the probe to bands containing sequences from small exons: for example exon 4 and exon 5 of the MBP gene are only 36 bp and 32 bp, respectively, c) partial

degradation of the gel-purified probe. These factors, however, should only have affected hybridization intensity and should not have affected the detection of the 3.0 kb PvuII bands. The PvuII polymorphism maps around exon 1 which is a long exon. Furthermore, there was very little lane to lane variation between bands of same MW, as evidenced from the intensity of the 2.2 kb-2.1 kb PvuII doublets. It was always possible to distinguish between the complete absence of a band and the presence of a very weak band. When necessary, sample analysis was repeated and further confirmed using larger amounts of DNA. All the blots were blindly read by three different observers.

The patterns of the HincII digests (Fig. 18) were sometimes complicated by partial enzyme digestions. Again, detection of the polymorphic variant was not affected. Partial digestion affected specifically the high MW bands and in Fig. 18 for example, where 4 DNA samples (samples 65 to 68) showed partially digested bands, the 1.0 kb band was still clearly observed in DNA sample 65.

Table II and Table III showed the presence of 3.0 kb PVUII and 1.0 kb HincII bands in 12 out of 34 MS patients, a proportion of 35.3%. Among the 34 healthy controls examined, the minor polymorphic variants were found in 9 DNA samples, *i.e.* 26.5%. This percentage was not statistically different from the incidence of rare polymorphism among MS patients. A

relative incidence or risk factor of only 1.5 showed some slight increase of rare polymorphism among MS patients as compared to controls but certainly did not indicate definite association between rare PvuII-HincII polymorphic variants and MS incidence. The 3.0 kb PvuII and 1.0 kb HincII variants, when considered alone, are therefore, not among the important factors that predispose some individuals to or protect them against MS. Absence of relationship between those DNA polymorphisms and the sex of MS patients was to be expected since the MBP gene does not map on the X chromosome. It is unlikely that polymorphisms on an autosomal chromosome will affect another gene on the X chromosome (such as the PLP gene) which itself could be associated with MS.

The PvuII-HincII polymorphism in the 5' flanking region of the MBP gene did not influence the disease pattern in MS patients at least when the patterns are classified as inactive, progressive or relapsing-remitting chronic MS. These polymorphisms appeared also independent of the age of MS onset, although more patients with MS onset after 40 years of age might have to be analyzed for confirmation. It is interesting to note that polymorphism frequencies measured for MS patients as a group (35.3%), for the patients with progressive MS (35.0%), for the group of patients with relapsing MS (25.0%), for MS patients with disease onset before 25 years (35.7%) or between 25 and 40 years of age (29.4%) were all in the same range as the frequency measured

among the 34 healthy individuals, controls for MS group (26.5%) or the frequency measured for placental donors (30.0%) or random blood donors (30.0%). Although these frequencies were not statistically different from each other, the frequency in MS patients as a group (35.3%) and the frequencies among patients with progressive MS (35%) or among patients developing MS before 25 years of age (35.7%) were slightly higher. A 30% frequency for polymorphisms that might be linked to an often fatal disease may seem very high, however, these polymorphisms should be viewed only as one element, acting in conjunction with other genetic factors and environmental factors.

The length polymorphisms analyzed in this study seem different from the length polymorphisms found in flanking regions of several genes, which have all been found associated with some disease (reviewed by Nakamura *et al.*, 1987). For example, unique alleles of the tandem repeat RFLP in the 5' flanking region of the c-Ha-ras oncogene have been frequently found in cancer patients and tumor DNA, but not in individuals without a cancer or a family history of cancer (Colb *et al.*, 1986; Krontiris *et al.*, 1986). Similarly, length polymorphisms 5' to the human insulin gene have been associated with non-insulin dependent diabetes mellitus (Hitman *et al.*, 1984; Rotwein *et al.*, 1986), with insulin-dependent diabetes mellius (Bell *et al.*, 1985) and even with atherosclerosis (Mandrup-Poulsen *et al.*, 1984).

The data showing non association between polymorphisms in the 5' flanking region of the MBP gene and susceptibility to MS, do not exclude the MBP gene and the surrounding genome as genetic factors affecting MS incidence. Since the MBP gene is long and appeared highly polymorphic, other DNA changes within the gene might well play a role in susceptibility to MS. The polymorphisms detected in this study also constitute an additional marker, like the peptidase A gene, for chromosome 18 and particularly the 18q22-q23 region where several chromosomal abnormalities (trisomies) have been found previously (Turleau and de Grouchy, 1977; de Muelenaere *et al.*, 1981).

CHAPTER 6

SUMMARY

A human brain c-DNA library was screaned for MBP sequences using a rat c-DNA probe. Two c-DNA fragments, a 1.2 kb EcoRI and a 1.0 kb EcoRI were purified. These fragments together must constitute the full length human MBP c-DNA for the following reasons:

- their sizes added to 2.2-2.3 kb, the full size of the human MBP c-DNA.

- the 1.0 kb fragment always hybridized very weakly to rat MBP sequences while the 1.2 kb fragment which must contain the coding sequence did hybridize strongly.

- the partial restriction map was consistent with the published sequence of the human MBP c-DNA i.e. 1 BamH1 site, 1 PstI site, 2 PvuII sites all within the 1.2 kb EcoRI c-DNA portion and 2 XbaI sites within the 1.0 kb c-DNA portion.

Synthetic oligonucleotide probes based on the human MBP sequence yielded only limited information on the gene: a 5.8 kb EcoRI and a 4.0 kb BamH1 were part of the third or fourth exon of the gene. The rat c-DNA probe was, therefore, used to analyze the human MBP gene. When comparing the hybridization patterns of DNA from different unrelated individuals, DNA polymorphic changes affecting several enzymes and mapping in at least 2 different loci were detected:

- some polymorphisms were detected with enzymes BamH1, BclI, BglII, EcoRI.and PvuII; these affected 3 or 4 DNA

fragments in each enzyme digest and the alleles involved always differed from each other by 300-400 bp indicating insertion-deletion as the mutation affecting all the above enzyme sites. Because a 5.8 kb EcoRI and a 4.0 kb BamHl fragments were among the alleles in these polymorphisms, these length polymorphisms must map within the gene, near the third or fourth exon.

- another type of polymorphism was revealed by enzyme PvuII; this involved PvuII fragments of size 3.6-3.5 kb and 3.1-3.0 kb which, based on published data, mapped on the 5' flanking region. This restriction length polymorphism also generated 1.5 kb SalI and 1.0 kb HincII alleles in some individuals.

- a third DNA polymorphism change might involve additional PvuII sites and generate 4.3 kb and 3.8 kb PvuII allelic fragments.

Polymorphisms in the 5' flanking region of the MBP gene were studied in more detail and for a general population, frequencies of 30.0% (6 placental DNA out of 20 or 3 blood donors out of 10) were obtained for the presence of the 3.0 kb PvuII and the 1.0 kb HincII alleles.

In a pilot study involving 34 chronic MS patients and 34 matched-controls, the minor PvuII-HincII polymorphic alleles appeared in 35.3% of MS patients and in 26.1% of controls suggesting no clear linkage between polymorphism and susceptibility to MS. There was also no correlation between polymorphisms in the 5' flanking region of the MBP gene and

sex of MS patients, pattern of disease in chronic MS patients or age of MS onset. However, because of the multifactorial nature of MS, an association between polymorphism and disease risk cannot be classified as definitively positive or negative. Polymorphic variants which alone show no disease association may still constitute risk factors when studied in combination with other polymorphisms elsewhere in the genome or other factors. The data presented here did not completely rule out the possibility of an association between the documented or other DNA polymorphisms within the MBP gene and various MS cases.

A direct linkage between the PvuII-HincII lenght polymorphism of the MBP gene and acute MS was not determined.

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