THE GENES FOR MYELIN BASIC PROTEIN IN NORMAL AND SHIVERER MUTANT MICE

Thesis by
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The support and freedom that my parents have always given me ultimately made this possible, and I am proud to have learned from their lessons and their examples.

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ABSTRACT

A cDNA library was constructed from the brains of 13 day old rats, and was screened with a synthetic DNA probe to yield clones representing myelin basic protein (MBP). One 1.9 kb clone was sequenced and found to encode the 14 kd MBP. Using this clone as a hybridization probe, cosmid clones from a library of wild type mouse DNA were selected and characterized. One clone was shown to carry five exons which encode 14 kd MBP, distributed over a 32 kb region. A sixth exon was detected with a synthetic DNA probe, and was found to encode the 41 amino acids which distinguish 18.5 kd from 14 kd MBP. The 5' end of the gene was mapped with S1 nuclease protection and primer extension experiments to a position 47 bp 5' of the initator codon for MBP synthesis. It was shown that the gene cloned is probably the only MBP gene in the mouse genome.

Cloned DNAs were used to analyze the MBP gene and its expression in the myelin deficient mutant mouse shiverer. It was shown that a deletion has removed five out of six MBP exons, leaving only the 5'-most exon and 13 kb of the first intervening sequence. The deletion completely prevents expression of normal 2.1 kb MBP mRNAs, but a 16-fold lower number of transcripts are observed which initiate correctly at the 5' end of the first exon, are not correctly spliced, and are rarely polyadenylated. If translated, they would direct synthesis of a 61 amino acid peptide containing the first 56 amino acids of MBP. The MBP gene was mapped to mouse chromosome 18 by hybridization of MBP probes with DNA from Chinese hamster-mouse hybrid cell lines, showing it to be linked to the shiverer mutation. It is proposed that the partial deletion of the MBP gene is the primary lesion of the shiverer mutation.

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INTRODUCTION

The Function of Myelin

Like most cells, neurons have a difference in electrical potential across their plasma membranes, such that the interior is negative with respect to the extracellular environment. This resting state of membrane polarization is generated by an arrangement of ion-selective conductances and a transmembrane ion pump.

In unmyelinated nerve fibers, impulses are conducted as a wave of transient depolarization of the plasma membrane which moves continuously down the length of the axon. The momentary depolarization at any point is due to ion fluxes through regulated, ion-specific transmembrane channels, which briefly disrupt locally the steady state condition of ion localization and membrane potential. The depolarization process spreads to successive points down the axon by local currents which induce a small depolarization ahead of the region of maximal depolarization. This small change in membrane potential initiates openings of local ion channels which then proceed to give rise to the full depolarization. This induces the adjacent membrane to initiate its own depolarization, and so on down the fiber. The duration of the depolarization at any point is about one millisecond, after which it is returned to the resting state.

Myelination increases both the speed and the efficiency of conduction of impulses (for review, see Rogart and Ritchie, 1977). Speed is limited by the distance down the axon over which a full depolarization at one point can induce openings of ion channels, and the time required for these channels to open. The distance over which a local depolarization can spread is limited by the resistance of the axoplasm, and the conductance and capacitance of the axonal membrane. The higher the capacitance, the more charges have to be moved to effect a given change in membrane potential, and therefore, the depolarization will extend over a shorter distance. Myelination greatly decreases the capacitance of a segment of

the axon, thereby allowing the depolarization to spread further and induce channel openings over a greater distance. In addition, ion channels are concentrated at the nodes of Ranvier in myelinated axons, greatly decreasing the membrane conductance of the myelinated internodal axon segment, and allowing the depolarization to span the myelinated internodes by rapid passive spread.

The energy required for this process derives from ion concentration differences across the axonal membrane, which are produced and maintained by an ATP-dependent ion pump situated in the plasma membrane. This ion pump moves Na^+ ions out of the axoplasm and K^+ ions into it against their respective chemical gradients. The opening of ion channels during transmission of an impulse allows Na^+ ions to enter the axoplasm and K^+ ions to leave it. The ion pump expends about 50% of the energy requirement of most neurons in restoring the ion gradients. By reducing the number of ion channels on a nerve fiber, myelination reduces the number of ions which must be pumped back and therefore increases the energy efficiency of the process.

An alternative strategy for increasing conduction velocity is to reduce axoplasmic resistance by increasing the diameter of the axon. To achieve the same conduction velocity as a myelinated frog fiber 12 μm in diameter, the squid giant axon must be 500 μm across. This strategy accomplishes an increase in speed of transmission, but it does so at the expense of space and energy efficiency.

Myelination

The time of onset of myelination differs from one region of the nervous system to the next, but generally the earliest regions begin before or at birth, and myelination continues for several weeks thereafter. Peripheral nervous system (PNS) myelination typically precedes that of the central nervous system (CNS) by several days.

Myelin is produced by glial cells, and is the result of an interaction between the neuron and either Schwann cells in the PNS or oligodendrocytes in the CNS. Numerous indications exist that controlling signals are produced by the neuron which help regulate myelin production but the nature of these remains the subject of investigation. Unlike the Schwann cell, which produces a single internode on one fiber, the oligodendrocyte produces many internodes, estimated as numbering 30-50, on many axons. Each internode is connected to the cell body by a cytoplasmic bridge. The formation of a myelinated internode begins as a cytoplasmic process is extended by the oligodendrocyte towards an axon, and begins to wrap around it, in concentric layers. The cytoplasm initially present in the process is extruded as the wrapping tightens or condenses, leaving only a thin strip at the perimeter of the sheet.

The loss of cytoplasm leaves concentric, tightly wrapped layers of a specialized plasma membrane. The apposition of the cytoplasmic faces of two membranes produces the major dense line viewed by electron microscopy, whereas the region of contact of adjacent outer surfaces produces the less prominent intraperiod line (for review, see Raine, 1977).

Myelin Basic Protein and its Role in Myelin

Studies on bulk myelin are usually carried out on density gradient-purified material from brain homogenates. For review, see Norton, 1977. In homogenized brain, myelin forms low density vesicles containing material from sheaths, contaminated with parts of their axons. Some of this latter material may be removed by further purification steps. The greatest purity is achieved when the most myelin is present to start with, and therefore it is difficult to determine accurate compositions of myelin from mutants deficient in myelin, or from early stages in

development when only small amounts of myelin have been deposited. Good purity is achieved from adult white matter, where myelin can constitute over 50% of the dry weight.

Purified myelin is 70% lipid and 30% protein by dry weight. The lipid composition differs from that of most cells, most notably in the high cerebroside and somewhat higher cholesterol levels, as well as there being an increase in very long chain fatty acids (Bourré, 1980).

The most abundant protein of CNS myelin is proteolipid protein (PLP), an integral membrane protein esterified with a fatty acid, with 60% nonpolar residues. Recently, Laursen *et al.* (1984) have proposed a transmembrane structure for PLP, with an extensive, basic cytoplasmic domain, several transmembrane segments, and a loop of hydrophobic residues at the outer surface which might be important in maintaining the close apposition of external membrane surfaces in compact myelin.

Second in abundance, comprising 30% of total protein in CNS myelin is MBP. For review, see Carnegie and Moore (1980). Some species, such as human and bovine, are believed to have a single, 18.5 kilodalton (kd) form of MBP, while mouse and rat have four highly-related forms. The sizes of the four forms are 21.5 kd, 18.5 kd, 17 kd and 14 kd. All are similar in composition, in that they have a high proportion of basic residues, distributed over the length of the polypeptide, giving the proteins an isoelectric point of greater than 10. The structure of the protein is not known, but it appears able to associate with negatively charged lipids, and to insert short loops into the hydrophobic portions of a lipid bilayer. Some self-aggregation of MBP under non-denaturing conditions has also been reported. The work presented in Chapter 2 indicates that all four forms in mouse are probably the product of a single gene.

While the role of MBP is not known, it is thought to be important in the maintenance of myelin structure. It has been localized to the major dense line of myelin, which represents the area of contact of two adjacent cytoplasmic faces of the membrane in the mature, condensed structure (Omlin *et al.*, 1982). Because of MBP's ability to interact with the chemical environment presented by the plasma membrane surface, stabilization of the lamellar structure is thought to occur by the MBP molecules associating with the cytoplasmic surfaces of each membrane at the major dense line, allowing the surfaces to come together by neutralizing the negative charges on the phospholipid head groups, and perhaps by a dimerization of the MBP's bound to the two surfaces.

The greatly reduced amount of central myelin, and the abnormal structure of the remaining sheaths in mice homozygous for the autosomal recessive shiverer mutation, which lack MBP as the result of a partial deletion of their MBP gene, supports the view that MBP is required for normal myelin morphology (Chapter 3).

Myelin Basic Protein and Multiple Sclerosis

Much of the study on MBP has been stimulated by the early observations that MBP is the antigen responsible for experimental allergic encephalomyelitis (EAE), a demyelinating syndrome resembling multiple sclerosis, which can be induced by immunizing experimental animals with brain tissue (Kabat *et al.*, 1946), myelin basic protein (Laatsch *et al.*, 1962; Kies and Alvord, 1959), or synthetic peptides corresponding to regions of the primary structure of MBP (Eylar *et al.*, 1970; Hashim *et al.*, 1978). In EAE both antibodies and a cellular immune response against MBP are observed, and plaques of demyelination, similar to those found in multiple sclerosis, are often observed. For review, see Paterson (1973). While it is clear that MBP is the important antigen in EAE, it is not known what role, if any, MBP plays in the etiology of multiple sclerosis.

The work presented in this thesis was initially undertaken with a view towards obtaining a cloned DNA probe for use in molecular genetic studies of multiple sclerosis patients and high risk groups, in collaboration with Drs. Stanley Prusiner and Kevin Boylan at the University of California, San Francisco, and for the study of the gene or genes for MBP and their expression. As will be seen, the work also expanded to include studies on the MBP gene of the shiverer mutant mouse.

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Characterization of Cloned cDNA Representing Rat Myelin Basic Protein: Absence of Expression in Brain of Shiverer Mutant Mice

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Summary

A cDNA library was constructed from mRNA isolated from the brains of 18-day-old rats, the age at which myelin biosynthesis is maximal. A synthetic DNA probe synthesized based on reverse translation of the amino acid sequence of rat myelin basic protein (MBP) was used to select two cDNA clones encoding MBP. A 1.5 kb Eco RI fragment from one clone was completely sequenced. When translated, a portion of this sequence was identical at 126 of 127 positions with the reported amino acid sequence for small MBP from the rat. Brains from mice of the homozygous shiverer genotype contained neatly reduced amounts of MBP mRNA relative to wild type. A deletion of MBP sequences in the genome of shiverer mice was also demonstrated. cDNAs for MBP will allow molecular investigation of the role this gene plays in both dysmyelinating and demyelinating diseases, as well as questions of MBP biosynthesis.

Introduction

Myelin surrounds axons and facilitates the conduction of nervous impulses. Myelin basic protein (MBP) is the major protein of central nervous system myelin and constitutes as much as 30% of the total protein. One MBP has been found in humans, cows, pigs, sheep, guinea pigs, and rabbits (Martenson et al., 1971a). Two MBPs have been found in rats, mice, and squirrels (Martenson et al., 1971b). The molecular weights (M_r) of the MBPs in rats are 14,000 and 18,500. Protein sequence analysis has shown that the small MBP of rat is identical with the large MBP except for a deletion of 40 amino acid residues near the C terminus (Dunkley and Carnegie, 1974; Martenson et al., 1972). The existence of two additional minor species in mouse also has been reported (Barbarese et al., 1977; Yu and Campagnoni, 1982). The amino acid sequence of MBP is highly conserved throughout these species (Martenson, 1980).

Although MBP is not known to possess enzymatic activity, it is thought to play an important structural role in the maintenance of myelin function. MBP is known to undergo acetylation, methylation, and phosphorylation (Martenson, 1980). Mutant mice with reduced levels of MBP exhibit profound neurological dysfunction (Baumann, 1980).

MBP may play an important role in demyelinating diseases. For many years, experimental allergic encephalomyelitis (EAE) has been studied as a model for multiple sclerosis (Paterson, 1973). EAE is primarily a demyelinating disease that was initially produced by immunizing laboratory animals with extracts of myelin (Rivers and Schwentker, 1935; Kabat et al., 1946; Morgan, 1946). The encephalitogenic factor in the myelin extracts was eventually shown to be MBP (Kies and Alvord, 1959; Laatsch et al., 1962). Subsequent studies demonstrated that a nonapeptide of bovine MBP (residues 113–121) could itself produce EAE in guinea pigs (Eylar et al., 1970). Larger fragments of MBP are required to produce EAE in other laboratory animals (Brostoff, 1977).

To begin investigating the regulatory mechanisms controlling the biosynthesis of MBPs as well as genetic factors involving dysmyelinating disorders and demyelinating diseases, we isolated a cDNA clone encoding rat MBP. A cDNA library was constructed from mRNA that was isolated from the brains of 18-day-old (postnatal) rats, the developmental period of maximal myelin biosynthesis (Norton and Poduslo, 1973). A synthetic DNA probe, deduced from reverse translation of the MBP amino acid sequence, was synthesized and used to select two cDNA clones encoding MBP. We report the nucleotide sequence of part of one MBP cDNA clone and preliminary Southern blots of genomic DNA. The data presented do not enable us to differentiate between the existence of separate genes for large and small MBPs and the existence of a single gene which gives rise to both proteins through differential processing. We also show that mutant mice homozygous for the shiverer mutation (Baumann, 1980) have profoundly reduced levels of MBP mRNA in their brains, a finding in accord with the observed depletion of MBP in shiverer myelin. Genomic blots of shiverer DNA show that these mice have lost much of their MBP mRNA coding regions.

Results

Strategy

The amino acid sequences of the rat MBPs have been determined (Dunkley and Carnegie, 1974; Martenson et al., 1972). Thus a set of sixteen 14-mers was synthesized based on the codons reverse translated from positions 78 to 82. Based on the amino acid sequence N-Gln-Asp-Glu-Asn-Pro-C, the DNA sequences 5′-GG(A/G)TT(T/C)TC(A/G)TC(T/C)TG were synthesized. This mixture of synthetic DNA probes was end labeled with ^{32}P and used to screen the 18-day rat brain cDNA library. Two cDNA clones were obtained and have been characterized by restriction map and DNA sequence analyses.

The Synthetic Oligonucleotides Detect a 2.1 kb mRNA

Total RNA from liver and poly(A)$^+$ RNAs from brain and liver of 18-day-old Sprague-Dawley rats were blotted onto nitrocellulose and hybridized with ^{32}P-labeled oligonucleotides (Fig. 1). Autoradiography revealed that the oligonu-

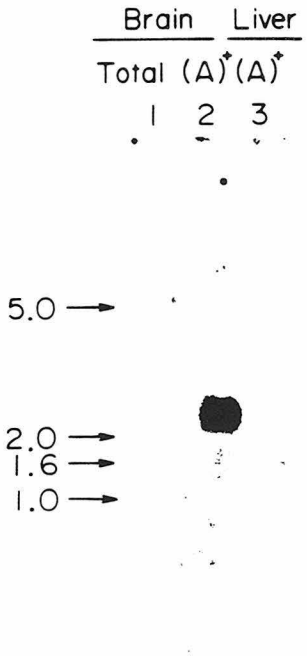


Figure 1. Blot of RNA from Brain and Liver of 18-Day-Old Rats Hybridized with the Synthetic MBP Oligonucleotide Probe

Lane 1, 5 μg total RNA from brain; lane 2, 2 μg poly(A)+ RNA from brain; lane 3, 1 μg poly(A)+ RNA from liver. Size markers are from rRNA and DNA restriction fragments.

cleotide pool recognized an RNA of 2.1 kb that is present in polyadenylated brain RNA but is undetectable in liver or total brain RNA. The weak bands at 5.0 kb and 2.0 kb in brain total RNA are the result of a low level of nonspecific hybridization to ribosomal RNAs. Thus the majority of the MBP appears to be encoded by mRNA of a single size class. With the limitations of the RNA blotting technique, we cannot resolve separate mRNA species that could encode large and small MBP. Northern blot analyses with the two cDNA clones have confirmed this observation (see below).

Two Distinct Rat MBP cDNA Clones Are Identified

A screen of the cDNA library with the synthetic oligonucleotide probe yielded two distinct clones, λRB3 and λRB7. The insert of the λRB3 clone is a single Eco RI fragment of 2.0 kb, while the λRB7 clone contains two Eco RI fragments of 1.45 and 0.50 kb. These three Eco RI fragments were subcloned into the Eco RI site of pBR322 to generate clones pMBP-2, pMBP-1, and pMBP-3 respectively, and restriction maps were determined (Figure 2). To determine whether the two Eco RI fragments carried by λRB7 are derived from reverse transcription of a single RNA or from tandem ligation of two independent cDNAs carrying terminal Eco RI linkers, the two Eco RI fragments were gel purified and used as hybridization probes against rat RNA. Both fragments hybridized with a 2.1 kb mRNA, as did the synthetic oligonucleotide (Figure 3). When used as hybridization probes in genomic blots, both Eco RI fragments recognize a 10 kb Bam HI fragment (data not shown). It is therefore highly probable that both Eco RI fragments from λRB7 arise from a single reverse transcript of a single RNA molecule which has an Eco RI site in its sequence. The purified Eco RI fragment from λRB3 also hybridized with a 2.1 kb RNA (Figure 3). The left end of pMBP-2 appears to carry a highly repeated DNA element, and therefore this clone is not a useful probe for genomic blots.

The restriction maps indicate that the inserts of clones λRB3 and λRB7 share all restriction sites over 1.2 kb of their lengths but have different left and right ends (Figure 2). The homologies implied by restriction maps are confirmed by blot hybridization using nick-translated insert from pMBP-2 against restriction digests of pMBP-1 and pMBP-2 (data not shown).

The transcripts that gave rise to these two cDNA clones could not have arisen simply from a single gene as the result of different initiation and polyadenylation sites, since there are differences in restriction sites between the extreme left end of the insert from λRB7 and the corresponding region of the λRB3 insert. It is possible that these two transcripts are generated from two similar genes which arose through duplication, or that a single gene produced both transcripts through differential RNA processing. The inbred nature of the Sprague-Dawley rat makes it unlikely that these transcripts arose from nonidentical allelic genes. It is interesting that two restriction enzyme sites present at the left end of λRB7 but missing in λRB3 are in the protein coding region of λRB7 (see below).

Clone λRB7 Encodes the Entire Small MBP Amino Acid Sequence

Since the synthetic oligonucleotide mixture hybridized with the 1.5 kb Eco RI fragment from λRB7 (data not shown), the complete DNA sequence of this fragment, subcloned in pMBP-1, was determined (Figure 4). Nucleotides 28 to 411 code for the small (M_r 14,000) rat MBP. The translated DNA sequence is identical at 126 of 127 positions with the reported protein sequence of small MBP isolated from buffalo rat (Dunkley and Carnegie, 1974). The codon for alanine at position 1 is immediately preceded by ATG, coding for methionine. It therefore is possible that the N terminus of mature small MBP is generated directly by removal of the initiator methionine followed by N-acetylation of the adjacent alanine residue. The codon for arginine 127 is immediately followed by the termination codon TGA, indicating that the C terminus of MBP is generated without processing. Two more in-frame termination codons are found 17 and 20 codons farther downstream. Position 124 is methionine in the published protein sequence, but is translated as isoleucine in the cDNA sequence. A DNA

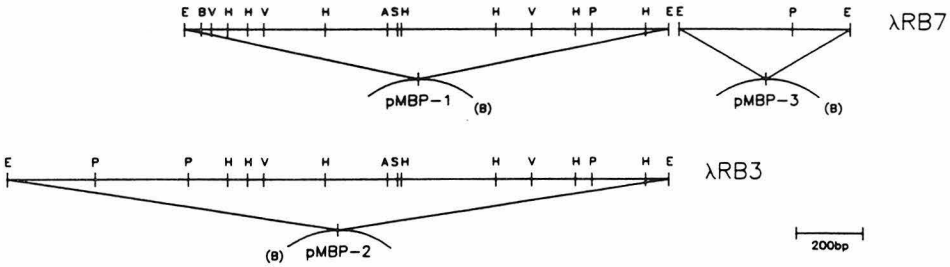


Figure 2. Restriction Maps of MBP cDNA Subclones pMBP-1, pMBP-2, and pMBP-3

E: Eco RI. B: Bam HI. A: Ava I. H: Hind III. P: Pst I. V: Ava II. S: Sal I. (B) indicates the direction of the vector Bam HI site from the Eco RI site of pBR322 as drawn.

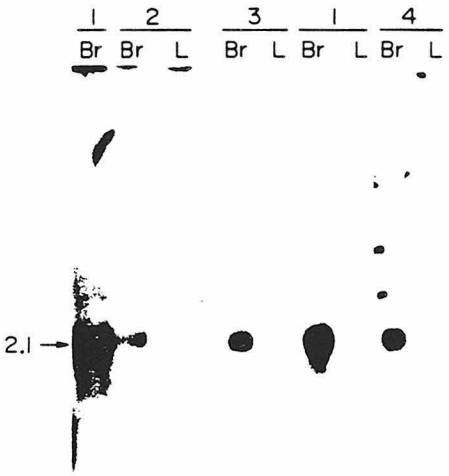


Figure 3. Both Eco RI Fragments from Clone λRB7 and the Insert from Clone λRB3 Recognize 2.1 kb Brain-Specific RNAs

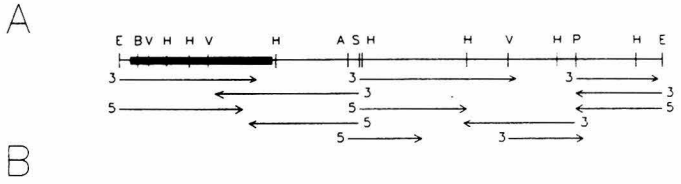
Poly(A)$^+$ RNAs from rat brain (Br) or liver (L) were blotted as described and hybridized with the following probes : (1) 1.45 kb Eco RI fragment from λRB7; (2) 0.5 kb Eco RI fragment from λRB7; (3) 2.0 kb Eco RI fragment from λRB3; (4) mixture of synthetic 14-mers. Probes 1-3 were labeled by nick translation; probe 4 by kinase.

sequence error is unlikely since the sequence at this position is unambiguous as determined on both strands of the cDNA fragment. There are at least two possible explanations for this difference. Reverse transcriptase errors occur at an approximate frequency of 10^{-3} (Sirover and Loeb, 1977) and may account for this difference. A second possibility is that this difference represents a genetic polymorphism which distinguishes the MBP genes of Sprague-Dawley and buffalo rats. Since a change of a single base pair can account for substitution of isoleucine for methionine, and since these amino acids are chemically similar, we feel that this difference is likely to arise from genetic polymorphism.

The open reading frame coding for small MBP extends 27 bp 5′ of the codon for alanine 1, to the 5′ end of the 1.45 kb Eco RI fragment of λRB7. We do not know how far 5′ of the Eco RI site this open reading frame extends or whether there may be another, upstream methionine from which translation is initiated. However, because of the cytoplasmic localization of MBP (Poduslo and Braun, 1975) and the direct appearance of mature MBP on cell-free translation of brain mRNA (Yu and Campagnoni, 1982), a leader peptide is not expected, and it is probable that translation is initiated at the methionine codon we have sequenced.

In addition to the open reading frame encoding MBP, an open reading frame of 579 bp exists in the insert of pMBP-1, from positions 608 to 1186 (Figure 4). There is no methionine codon in this open reading frame, but there is a sequence with good homology to the canonical splice acceptor sequence (Lewin, 1980) at positions 856-873. Assuming a Poisson distribution for lengths of open reading frames, and correcting for base composition of pMBP-1, we estimate the probability of finding an unselected open reading frame of this length in a 1.46 kb stretch of DNA as 0.07. While it is possible that DNA from this region serves as an exon in some other transcript, we detect only the 2.1 kb RNA in our RNA blots, and we know of no function for this sequence.

The sequence data for pMBP-1, derived from λRB7, also allow us to conclude that λRB3 does not encode large MBP. In adult rat brain, large MBP is present at approximately one-third the level of small MBP (Martenson et al., 1970). The 40 amino acid insertion that distinguishes large from small MBP is found between amino acids 114 and 115 of small MBP (Martenson et al., 1972). This position is encoded on a Hinf I restriction fragment of 233 bp (positions 204-437) in pMBP-1. λRB3 has this same 233 bp Hinf I fragment (Figure 2), which would have been replaced by one 120 bp larger if λRB3 were derived from the mRNA for large MBP. We therefore conclude that neither of the distinct cDNA clones isolated encodes large MPB.

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                                  Met Ala Ser Gln Lys Arg Pro Ser Gln Arg His Gly Ser Lys Tyr Leu Ala Thr Ala Ser Thr Met Asp His
1   TTCGGCTTCCGAAGGCCTGATGTG ATG GCA TCA CAG AAG AGA CCC TCA CAG CGA CAC GGA TCC AAG TAC TTG GCC ACA GCA AGT ACC ATG GAC CAT   96

                Ala Arg His Gly Phe Leu Pro Arg His Arg Asp Thr Gly Ile Leu Asp Ser Ile Gly Arg Phe Phe Ser Gly Asp Arg Gly Ala Pro Lys
97  GCC CGG CAT GGC TTC CTC CCA AGG CAC AGA GAC ACG GGC ATC CTT GAC TCC ATC GGG CGC TTC TTT AGC GGT GAC AGG GGT GCG CCC AAG  186

                Arg Gly Ser Gly Lys Asp Ser His Thr Arg Thr Thr His Tyr Gly Ser Leu Pro Gln Lys Ser Gln Arg Thr Gln Asp Glu Asn Pro Val
187 CGG GGC TCT GGC AAG GAC TCA CAC ACA AGA ACT ACC CAC TAC GGC TCC CTG CCC CAG AAG TCG CAG AGG ACC CAA GAT GAA AAC CCA GTA  276

                Val His Phe Phe Lys Asn Ile Val Thr Pro Arg Thr Pro Pro Pro Ser Gln Gly Lys Gly Arg Gly Leu Ser Leu Ser Arg Phe Ser Trp
277 GTC CAC TTC TTC AAG AAC ATT GTG ACA CCT CGT ACA CCC CCT CCA TCC CAA GGA AAG GGG AGA GGC CTG TCC CTC AGC AGA TTT AGC TGG  366

                Gly Gly Arg Asp Ser Arg Ser Gly Ser Pro Ile Ala Arg Arg Tnn
367 GGA GGA AGA GAC AGC CGC TCT GGA TCT CCC ATA GCA AGA CGC TGA GAGCCTCCCTGCTCAGCCTTCCCGAATCCTGCCCTCGGCTTCTTAATATAACTGCCTTAA  471

472 ACGTTTAATTCTACTTGCACCAAATAGCTAGTTAGAGCAGACCCTCTCTTAATCCCGTGGGGCTGTGAACGCGGCGGGGCCAGCCCACGGCACCCTGACTGGCTAAAACTGTTTGTCCCTT  591

592 TTTATTTGAAGATTGAGTTTCCTCGGGGTCTTCTCTGCCCCGACTTGCTCCCCGTGTACCTTGGTCGACTCCGGAGGTTCAGGTGCACGGACACCCTTTCAAGTTCACCCCTACTCCATC  711

712 CTCAGACTTTCTTTTCACGGCGAGGCGCACCCCTCCAGCTTCCGTGGGCACTGCGGATAGACAGGCACACCGCCAAGGAGCCAGAGAGCATGGCGCAGGGGACTATGTGGTCCAGGCTTC  831

832 CTTTGTTTTCTTTCCCCTAAAGAGCTTTGTTTTTCCTGACAGGATCAGACAGTCTTGGAGTGGCTTACACAACGGGGGCTTGTGGTATGTGAGCACAGGTCGGGCAGCTGTGAGAGTCCA  951

952 GAGTGGGGTGGCCCTGGGGACGCTTCCAGGCCAGCGGTTCCCTGCACCCCACCAGCTGATTTCGAGCGTGGCAGAGGGAAGGAAAGGGGCGAGCGGGCTGGGCAATGGACCCGACAGGAA 1071

1072 ACGGGGACTTAGGGGAACACGCTGGAGATGCCATGTGTGGCTGCCGAAGGTCACCATCTCTCCTCAGTGCTCCCCAGAGCAGGTGCTTTTAAGAACCCTGTTTCCTCTCAGAGCCTAGGG 1191

1192 AGAGTCCAAGGACATGGCGCATCAGGAAGTGGGACTGCAGGAGTTCTCTGGTGGCCTCGTGCTGTCCCTCTGGCCACTTCTCACTTTAGGGTGGTCACGGCAGCTCGCCATGGCAGTGCC 1311

1312 ATTGGTGCACACTAACCTCATGGAAAAGTAACCATTCCCTGCCTCTTAGAAAGAACTCATTCTTAGTTTTAGGAGGGTTCCTGTCGCTGAATCAAGTCGCTGCCCTGATGCAGGGCTGGC 1431

1432 CTTGGCGACCCTCCAGGGATGAGGAGCTCAGAA
```

Figure 4. DNA Sequence of the 1.45 kb Eco RI MBP cDNA Fragment from pMBP-1

(A) Sequencing strategy. Box represents translated region. Arrows indicate length of sequence read from a 3′ labeled or 5′ labeled end, as noted. (B) DNA sequence. Positions 28–408 encode small MBP. Underlining indicates region with homology to synthetic DNA probe.

Southern Blot Analysis of Rat MBP Genes

The existence of large and small MBP and their high degree of amino acid sequence homology prompted us to ask whether multiple MBP genes can be detected in the rat genome by Southern blot analysis. Our results indicate that 3′ untranslated sequences of λRB7 appear to be single copy, while sequences homologous to a restriction fragment from the cDNA clone containing the entire coding sequence for small MBP are present in multiple copies in the rat genome or are interrupted extensively by introns. Sprague-Dawley rat liver DNA was digested with restriction enzymes Eco RI, Bam HI, and Pst I, electrophoresed through 0.8% agarose gels, transferred to nitrocellulose, and hybridized with radioactively labeled cDNA restriction fragments subcloned from λRB7 (Figure 5). The 0.8 kb Eco RI–Ava I fragment from pMBP-1 carries 3′ untranslated sequences exclusively and has a Pst site 234 bp from its 3′ end. When used as probe against liver DNA, it detected two Pst I fragments (1.0 kb and 0.75 kb), a single Eco RI fragment (4.0 kb), and one strong Bam HI fragment (10 kb), as well as a second weak Bam HI fragment of 11 kb. This second band may be the result of incomplete Bam HI digestion since it is present at the same relative intensity when other probes are used. We believe it unlikely that it represents a second, partially homologous sequence because no such band is observed with either Pst I or Eco RI. We find these patterns consistent with a single copy of this 3′ untranslated region per haploid genome. Similar patterns result when a restriction fragment from pMBP-3 containing all the cDNA sequences from this subclone is used as probe (data not shown). The 0.6 kb Eco RI–Ava I fragment from pMBP-1 carries the entire coding sequence for small MBP as well as 200 bp of 3′ untranslated region and 24 bp 5′ of the methionine preceding alanine 1 of MBP. When used as probe against the restriction digests described above, a single band is never observed. There are two Pst I bands recognized by this probe (6.2 kb and 1.0 kb), two strong and one weak Eco RI bands (4.0, 3.9, and 4.25 kb), and Bam HI bands of 15, 12, 10, and 5.0 kb. Experiments in progress directed at cloning genomic MBP sequences will reveal whether the multiple bands observed in these digests result from multiple introns in a

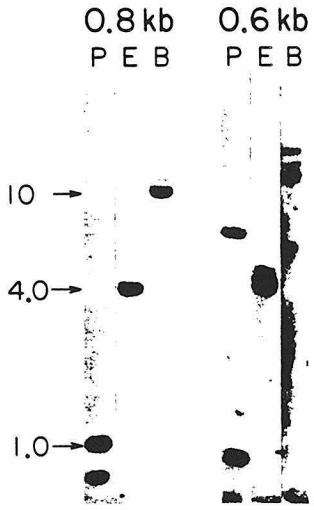

Figure 5. Southern Blots of Rat DNAs with MBP cDNA

Sprague-Dawley liver DNA (10 μg) was digested with the indicated restriction enzymes, electrophoresed through a 0.8% agarose gel, and blotted onto nitrocellulose. Probes used were as follows: (0.8 kb) 0.8 kb Eco RI–Ava I fragment of pMBP-1; (0.6 kb) 0.6 kb Eco RI–Ava I fragment from pMBP-1 containing entire MBP coding region. Restriction enzymes: (P) Pst I; (E) Eco RI; (B) Bam HI. Sizes are in kilobase pairs. Examination of original autoradiograms from two independent experiments confirms the interpretation of band patterns presented in the text.

single gene or from the existence of several copies of a sequence homologous to the cDNA probe fragment, possibly the result of separate small and large MBP genes.

MBP RNA in Shiverer Mice

Shiverer (shi) is a recessive autosomal mutation of the mouse, of unknown chromosomal location. Homozygous shiverer mice initially appear normal, but by 2 weeks after birth they develop a shivering motion that increases in severity and is eventually accompanied by paralysis and seizures. These mice die prematurely, between 50 and 100 days, but are fertile (Bird et al., 1977). Biochemical studies of homozygous shiverer mice show that the level of myelin basic protein in both central and peripheral nervous systems is less than 1% of control values. While central myelin shows serious morphological abnormalities, peripheral myelin appears normal (Dupouey et al., 1979; Kirschner and Ganser, 1980). RNA was purified from the brains of normal C57BL/6 and homozygous shi/shi adult mice for analysis of MBP-coding sequences. Twenty micrograms of total brain RNA from a normal mouse and two individual shi/shi mice, as well as five micrograms of total RNA from 18-day rat brain, were electrophoresed through a denaturing 1% agarose, 10 mM methylmercury hydroxide gel, and blotted onto nitrocellulose as described. The blot was probed with nick-translated 1.5 kb Eco RI fragment from pMBP-1. Lanes with both rat and normal mouse brain RNA contain a predominant 2.1 kb transcript complementary to the MBP probe, as well as a distribution of heterogeneous, lower molecular weight RNA presumed to be degradation products (Figure 6). RNAs from the brains of two shi/shi mice contained no detectable MBP-hybridizing transcripts. Comparisons of several exposures indicate that less than 1% of the normal level of MBP RNA would have been detected; therefore, we, conclude that brains from homozygous shiverer mice contain less than 1% of the amount of MBP-hybridizing RNA present in C57BL/6 brain. One effect of the shiverer mutation is to reduce dramatically the accumulation of MBP mRNA in the brains of homozygous mice, leading to the severe depletion of MBP observed biochemically. Further studies are required to determine whether the reduction or absence of MBP RNA sequences is the result of altered transcriptional rate or altered messenger stability, and whether this is the primary effect of the shiverer mutation.

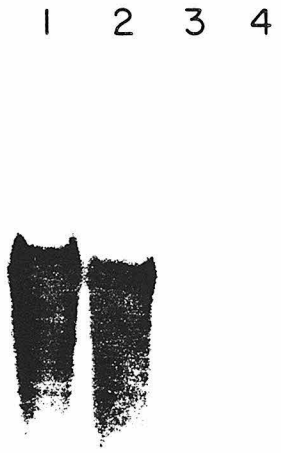

Figure 6. Blot of RNAs in Shiverer Mice Hybridized with MBP cDNA

RNA was electrophoresed through a 1% agarose 10 mM CH_3HgOH gel and blotted onto nitrocellulose. The blot was probed with nick-translated 1.5 kb Eco RI fragment from pMBP-1. Lane 1, 5 μg total RNA from 18-day rat brain; lane 2, 20 μg total RNA from C57BL-6 mouse brain; lanes 3 and 4, 20 μg total RNA from two individual shi/shi mice.

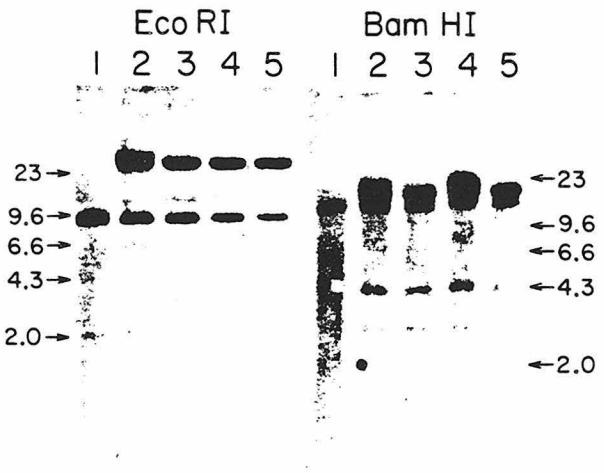

Figure 7. Southern Blots of DNAs from Shiverer and Other Mice with MBP cDNA

Mouse liver DNA (10 μg) was digested with the indicated restriction enzyme, electrophoresed through a 0.75% agarose gel, blotted, and hybridized with nick-translated 1.5 kb Eco RI fragment from pMBP-1. Lanes 1, shiverer; lanes 2, C57BL/6; lanes 3, AKR; lanes 4, SWR; lanes 5, BALB/c. Marker sizes are given in kilobase pairs.

MBP Genes in Shiverer Mice

Liver DNA was isolated from four normal strains of mice (C57BL/6, AKR, SWR, and BALB/c) and homozygous shiverer mice and was used in genomic blotting experiments with the Eco RI fragment of pMBP-1 as probe (Figure 7). When digested with Eco RI, all four normal mouse DNAs contained two major hybridizing bands, of 28 kb and 8.0 kb. Shiverer DNA has only one strongly hybridizing Eco RI fragment, at 8.0 kb, indicating that all the MBP cDNA-hybridizing sequences from the 28 kb fragment have been deleted in shiverer mice. In addition, a weak band of 2.0 kb is visible with both normal and shiverer DNAs.

When digested with Bam HI, all four normal mouse DNAs contained hybridizing fragments of 20, 15, 4.3, and 2.9 kb. Shiverer DNA has the 15 kb fragment and has lost hybridizing sequences from the 20 kb fragment. The signal from the 4.3 kb and 2.9 kb fragments is near the limit of detection, and we cannot determine whether these sequences are present in shiverer DNA.

These data clearly show that shiverer mice have lost significant portions of their MBP gene or genes, relative to four normal mouse strains. We cannot yet conclude whether complete genes or only major portions of one or more genes have been lost.

Discussion

We have isolated a clone from a rat brain cDNA library encoding small MBP. On the basis of our sequence and Northern blot data, it appears that small MBP is generated directly without posttranslational cleavages from a 2.1 kb mRNA. A second cDNA clone is strikingly homologous to the first, but differs in the coding region and does not encode large MBP. Moreover, it also appears to have been encoded by a 2.1 kb mRNA. These two mRNAs, as well as that encoding large MBP, may arise from distinct but closely related genes or from a single gene by alternative patterns of RNA splicing.

Shiverer mice produce less than 1% of the normal levels of MBP. We have demonstrated that shiverer mice produce no detectable brain mRNA complementary to our MBP cDNA. Southern blot analyses of the DNAs from shiverer mice with our MBP cDNA demonstrate that several restriction fragments are missing when compared to the DNAs of other inbred mouse strains. These data do not distinguish between two hypotheses. The shiverer mouse may have a deletion of one of several MBP genes, or it may have deleted portions of a single MBP gene or genes.

The availability of a cloned MBP-specific probe will allow us to pursue questions of MBP biosynthesis previously inaccessible. Genomic DNA sequences encoding both large and small MBPs of mice, humans, and rats can be cloned and examined directly. A variety of mouse neurological mutants, including the shiverer mice, can now be studied at the nucleic acid level. The genetic contribution to human demyelinating diseases such as multiple sclerosis or Guillain-Barré Syndrome also can be probed.

Experimental Procedures

Synthetic Nucleotides

Synthetic oligonucleotides were synthesized on an automated DNA synthesizer using solid-phase phosphoramidite chemistry as previously described (Beaucage and Carruthers, 1981; Matteucci and Carruthers, 1980). The oligomers were separated from smaller incomplete reaction products by preparative polyacrylamide gel electrophoresis.

RNA Isolation

RNA was extracted from the brains and livers of 18-day-old Sprague-Dawley rats by homogenization in 5 M guanidinium thiocyanate, followed by ultracentrifugation of RNA through a 5.7 M CsCl cushion (Chirgwin et al., 1979). Poly(A)$^+$ RNAs were enriched for by oligo(dT) cellulose chromatography (Aviv and Leder, 1972).

Oligonucleotide Hybridizations

Purified oligonucleotides were labeled using γ-^{32}P-ATP and T4 polynucleotide kinase as previously described (Houghton et al., 1980). Nitrocellulose filters bearing nucleic acids for hybridization with the 14-mer mixture were prehybridized in 5× SET, 5× Denhardt's solution (Denhardt, 1966), and 200 μg/ml sheared salmon sperm DNA at 37°C for 4 hr. Hybridization was in a solution of the same composition, supplemented to 5×10^5 cpm/ml (0.1 pmole/ml) with ^{32}P-labeled 14-mer mixture, at 37°C for 24 hr. Filters were washed in 5× SET, 0.05% sodium pyrophosphate, at 5°C, and autoradiographed. 1× SET is 0.15 M NaCl; 0.030 M Tris, pH 7.8; 0.001 M EDTA.

cDNA Library Construction

Double-stranded cDNA was synthesized from rat brain RNA by standard methods, by using oligo(dT) as a primer and low concentration S1 nuclease digestion to cut the single-stranded loop generated during self-priming of the second strand (Efstratiadis et al., 1976). The double-stranded cDNA was size-fractionated by BioGel A150m column chromatography, and material of average size 1.5 kb (range 0.5–4.5 kb) was selected. After treatment with Eco RI methylase, the cDNA was cloned into the *imm*^{434}, Eco RI insertion vector λgt10 (T. Huynh and R. Davis, personal communication), using Eco RI linkers. Growth of the in vitro packaged phages on an *hfl* host enriches for insert-carrying phages. Forty nanograms of double-stranded cDNA resulted in a library of 7.5×10^4 plaques, of which approximately half carried inserts. The library was screened (Benton and Davis, 1977) using hybridization conditions described above. Some potential positive cDNA clones were rescreened, and the DNAs from several of the confirmed positive clones were prepared (Davis et al., 1980). These inserts were subcloned into the Eco RI site of pBR322 as previously described (Dugaiczyk et al., 1975).

DNA Sequence Analysis

DNA sequences were determined by the Maxam-Gilbert method, as previously described (Maxam and Gilbert, 1980).

Restriction Mapping

Restriction maps of clones were determined by single and multiple digests with commercially available restriction enzymes, or by the method of Smith and Birnstiel (1976).

Southern and RNA Blot Analyses

Genomic DNA was prepared by the method of Blin and Stafford (1976). Southern and Northern blots were carried out as previously described (Southern, 1975; Thomas, 1980). Probes were either nick translated (Rigby et al., 1977) or end labeled (Maxam and Gilbert, 1980) DNA fragments.

Acknowledgments

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Note Added in Proof

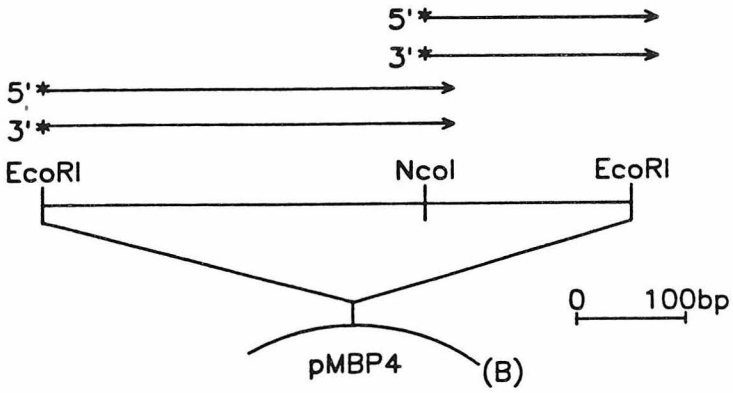
Genomic blots performed using liver DNA from mice of the SWV strain, in which the shiverer mutation arose, gave MBP-hybridizing band patterns identical with those observed for the wild-type strain, as shown in Figure 7. We are grateful to D. Juriloff for the gift of four SWV mice.

Appendix to Chapter 1

THE DNA SEQUENCE OF THE cDNA INSERT OF CLONE pMBP-3

The cDNA insert of the MBP clone λRB7 was subcloned into pBR322 as two EcoRI fragments of lengths 1462 bp (pMPB-1) and 550 bp (pMBP-3) (Figure 2). The sequence of the larger fragment is presented in Fig. 4, and contains the entire coding region for 14 kd MBP. The sequence of the cDNA insert of pMBP-3 was determined and is presented in Figure 8. The sequence consists of 425 bp of 3' untranslated material followed by more than 100 A residues. DNA molecules in plasmid pMBP-3 DNA have a distribution of lengths of poly(A) tracts all longer than 100, presumably as the result of infrequent small deletions during replication of this region. Therefore, the cDNA copy of rat 14 kd MBP mRNA cloned in λRB7 extends to the 3' extreme of the transcribed region. The numbering of nucleotides is continous with the numbering of pMBP-1 in Figure 4. Six to 20 base pairs 5' of the beginning of the poly(A) tail three tandem poly(A) addition consenses sequences underlined (Proudfoot and Brownlee, 1976). This additional information completes the sequence of the parent cDNA clone λRB7, consisting of 1,885 base pairs of cDNA sequence and a 3' tail of over 100 A residues.

Figure 8. Sequence of the cDNA insert of pMBP-3. The nucleotide sequence was determined by the method of Maxan and Gilbert (1980). Arrows indicate sequence read from a restriction fragment end-labeled at the 5' or 3' end, as indicated, at the site indicated by an asterisk. The EcoRI site at the left end of the clone as drawn derives from rat MBP sequences, while the one at the right is from the EcoRI linkers used in the library construction. The NcoI site used in labeling is underlined, at position 1798. Tandem AATAAA signals are underlined, positions 1866 to 1880.

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1461  AATTCCAGTCTTCTAATGTCCACGGACACCTCCCCATCCCTCTAACGTACTGACTATGTC  1520
1521  TTTTGATTTAGCATGTCTTCTATAGACCTTCCAAAGAGACCCACACTGGCACTGTCACCC  1580
1581  CCTAGGAGGGAAGGTGATGGTTGATATAGCCCGACGCGCATCTTGTTAATCCGTTCTAAT  1640
1641  TCCGAGGAGAGTGTGGGTTTAAGATAACACCTATTAAGTCATTGCCACAATAATGTGGGG  1700
1701  GTAAGAGAAACGCAGGGACGAAACTTCCAGAAACAAACCCTCCAGATCGTTCCACAGGAG  1760
1761  TGTTCGCCCTCCGGTGTGACTGAACGACCGACCTTGCCCATGGCTCATCCAGACAGCACA  1820
1821  GCTGCAGTATGGCTGGACAGAAGCACCTACTGTTCTTGGATATTGAAATAAAATAATAAA  1880
1881  CTTGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1940
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Chapter 2.

Cloning and Characterization of the Myelin Basic Protein Gene from Wild Type Mice: One Gene Can Encode Both 14 kd and 18.5 kd MBPs by Alternate Splicing of Transcripts

Introduction

The work presented in Chapter 1 produced a cloned rat cDNA probe for MBP which hybridizes strongly with mouse MBP sequences. It also indicated that a deletion at the MBP locus of the shiverer mutant mouse greatly reduces or abolishes expression of MBP. In this chapter the cloned cDNA probe is used further in the study of the mouse system.

While the human and bovine species are believed to have a single MBP species of 18.5 kd, mouse and rat have four forms of sizes 14 kd, 17 kd, 18.5 kd and 21.5 kd (Yu and Campagnoni, 1982; Agrawal et al., 1982). The interesting relationships between the primary structures of these proteins suggests that they could all derive from a single gene.

The characterization of genomic MBP sequences presented here suggests that the different proteins are encoded from a single gene of length 32 kilobases by alternate splicing pathways for primary transcripts.

The study of genomic MBP sequences from the normal mouse will allow the molecular analysis of the mutant presented in Chapter 3.

Results

Shiverer mutant mice have been shown to carry a deletion of a major portion of their MBP sequences (Chapter 1). One goal in the characterization of a normal mouse MBP gene is to create a standard against which the mutant can be compared. Since the shiverer mutant arose on the Swiss Vancouver (SWV) background (Chernoff, 1981), DNA from this strain was used in construction of a cosmid library (see Experimental Procedures). The library was screened by hybridization with the rat cDNA clone pMBP-1 (Chapter 1), and two overlapping, hybridization-positive clones, cos13 and cos138, were analyzed in detail. The

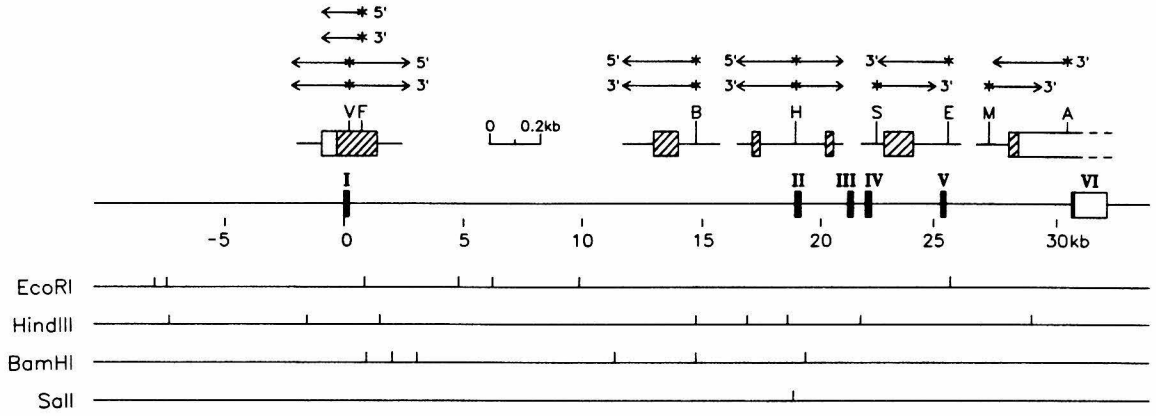
restriction map for the region represented by the two clones is presented in Figure 1. Clone cos13 extends from map position -9.6 kb to +27.6 kb, and clone cos138 extends from -4.0 kb to +33 kb.

Clone cos138 Carries a Complete Gene for Small MBP

Regions of clone cos138 complementary to a cDNA clone for rat 14 kd MBP (Chapter 1) were identified by hybridization of the nick-translated cDNA clone with gel blots of restriction digests of cos138 and of subclones carrying portions of the cos138 insert. The DNA sequences of the exons detected were determined and are presented in Figure 1. All of the exons shown are flanked by appropriate splice sites (Mount, 1982). Exon I encodes the first 56 amino acids of mouse MBP, which differ from the rat sequence at three positions. Exon II encodes amino acids 57 to 90, differing from the rat sequence at a single site. The 94 bp murine MBP cDNA clone isolated by Zeller et al. (1984) is identical in sequence to positions B6-B99 presented here. Exon III is only 36 bp in length and encodes amino acids 91 to 102, none of which differ from the rat sequence. Exon IV, 33 bp long, encodes amino acids 103 to 113, with no changes from the rat. Exon VI encodes amino acids 114 to 127 of mouse 14 kd MBP. However, the numbering of amino acid residues shown is for 18.5 kd MBP (see below), and therefore the amino acids encoded by exon VI are numbered 155 to 168. The entire sequence of exon VI has not been determined. The 5' end of the exon, encoding the C-terminal 14 amino acids of MBP, has been sequenced on both strands and shows a 3' splice site immediately followed by 42 base pairs of translated region encoding 14 amino acids, and a termination codon. As in the rat cDNA sequence, there are two additional termination codons 17 and 20 codons downstream of the first. A total of 0.86 kb of the 1.5 kb of 3' untranslated sequence has been determined on one strand, and found to be 94% homologous to the corresponding sequence from the rat cDNA sequence (data not shown). The 3' end of the exon has been determined

Figure 1. Sequencing of MBP-coding exons from clone cos138. Solid boxes numbered with roman numerals represent exons I through VI and show correct spacing, over 32 kb of the gene. Widths of the small exons are not to scale on this line. Solid black areas are protein coding, solid white are untranslated. Diagonally-striped boxes above show these same exons on a larger scale, as indicated, with restriction sites used for end-labeling. Solid white portions are untranslated. Restriction site codes are A, AvaI; B, BamHI; E, EcoRI; F, HinfI; H, HindIII; M, MspI; S, SalI; V, AvaII. Arrows above striped boxes indicate the Maxam-Gilbert sequencing runs for restriction fragments 5' or 3' end-labeled, as shown, at the ends indicated by the asterisks. The positions of restriction sites for Eco RI, HindIII, BamHI and SalI are shown below the small scale exon map.

The complete DNA sequences of exons I, II, III, IV and V are given, along with some of their flanking sequences. Only the first 117 nucleotides of exon VI are presented here. Nucleotide positions are shown below the DNA sequences, with nucleotide A1 corresponding to position 0 of the structural map. Amino acids are numbered above their three letter codes, beginning with the amino terminal alanine encoded by exon I, through to the C-terminal arginine encoded by exon VI. The numbering is for 18.5 kd MBP. Exon I is from nucleotide position A1 to A218, exon II from B1 to B103, exon III from C1 to C36, exon IV from D1 to D33, exon V from E1 to E123 and exon VI starts at F1, and continues for 1.5 kb. Features marked are: the 5' end of exon I (vertical arrow at position A1); TATA homology, underlined (A-33 to A-28); CCAAT homology, underlined (A-85 to A-80); oligonucleotide hybridizing region of exon V, underlined (E64 to E80); two in-frame termination codons downstream of the normally used one, underlined (F94 and F103).



EXON I

```
GGCCTAGGGAACCGCCCCCCACTTGATCCGCCTCTTTTCCCGAGATGCCCCGGGAAGGGAGGACAACACCTTCAAAGACAGGCCCTCAGAGTCCGACGAGCTTCAGACCATCCAAGAAGAC
A-100          A-80          A-60          A-40          10          A-20          A1          20
                                Met Ala Ser Gln Lys Arg Pro Ser Gln Arg Ser Lys Tyr Leu Ala Thr Ala Ser Thr Met Asp His
CCCACAGCAGCTCCGGAGGCTTGGATGTG ATG GCA TCA CAG AAG AGA CCC TCA CAG CGA TCC AAG TAC CTG GCC ACA GCA AGT ACC ATG GAC CAT
A20          A40          30          A60          A80          A100          50
Ala Arg His Gly Phe Leu Pro Arg His Arg Asp Thr Gly Ile Leu Asp Ser Ile Gly Arg Phe Phe Ser Gly Asp Arg Gly Ala Pro Lys
GCC AGG CAT GGC TTC CTC CCA AGG CAC AGA GAC ACG GGC ATC CTT GAC TCC ATC GGG CGC TTC TTT AGC GGT GAC AGG GGT GCG CCC AAG
      AI20          56          AI40          AI60          AI80          A200
Arg Gly Ser Gly Lys
CGG GGC TCT GGC AAG GTGAGCTCCGAGCCGTAGAGAAGCTGTGGGTTTAAATGCGG
```

EXON II

```
                                                                    57          60                    70
ACGCCCTCTCCATCCTCAGCTGCTCGCTTCTCTCTTTCAG GAC TCA CAC ACG AGA ACT ACC CAT TAT GGC TCC CTG CCC CAG AAG TCG CAG CAC GGC CGG
                                                                    BI                  B20                   B40                   B60
          80          90
Thr Gln Asp Glu Asn Pro Val Val His Phe Phe Lys Asn Ile
ACC CAA GAT GAA AAC CCA GTA GTC CAT TTC TTC AAG AAC ATT GTAAGTGACTGTGCACAGGGGAACCAAGAAATCATCAAGG
          B80                 B100
```

EXON III

```
                                                                    91                    100   102
GAAAGTGAGTCACTGCAGCCCACCCCTCTCTGTCTTCCAG GTG ACA CCT CGA ACA CCA CCT CCA TCC CAA GGG AAG GTAAGGCCTTGGATGTTTTGGTTCATCGAGA
                                                                    CI                  C20
```

EXON IV

```
                                                                    103                   110   113
CAATGTTTCTGTCGTCACTGTTTCTTTCTCTTCCTCACAG GGG AGA GGC CTG TCC CTC AGC AGA TTT AGC TGG GTAGGTGATGACTGCGTCTCCTCTGCTTCCTCATC
                                                                    DI                  D20
```

EXON V

```
                                  114                 120                                   130
AGCTCTGGTCTTTCTTGCAG GGG GCC GAG GGG CAG AAG CCA GGA TTT GGC TAC GGA GGC AGA GCT TCC GAC TAT AAA TCG GCT CAC AAG GGA TTC
                                  EI                  E20                 E40                   E60
Lys Gly Ala Tyr Asp Ala Gln Gly Thr Leu Ser Lys Ile Phe Lys Leu
AAG GGG GCC TAC GAC GCC CAG GGC ACG CTT TCC AAA ATC TTT AAG CTG GTAATGTAATATTCCTACCACGGGGAAACACCTGGGCACC
   E80                 E100                E120
```

EXON VI

```
                                                                    155         160                   168
CACAGGCTTGTGCCTAACTCCTGTCTTTTCTTTTTCCCAG GGA GGA AGA GAC AGC CGC TCT GGA TCT CCC ATG GCG AGA CGC TGA GAGCCCTCCCCGCTCAGCC
                                                                    FI                  F20                   F40
TTCCCGAATCCGCCCTCGGCTTCTTAATATAACTGCCTTAAACTTTTAATTCT.....
```

by DNA sequence comparison with the 3' end of the rat cDNA clone, and an S1 nuclease protection experiment (see below).

Therefore the mRNA for 14 kd MBP is, in the mouse, generated by splicing together five exons which are located over 32 kb of chromosomal DNA.

18.5 kd MBP Has an Additional Exon

While humans are believed to have only 18.5 kd MBP, mouse and rat have 14 kd, 17 kd, 18.5 kd and 21.5 kd forms (Barbarese et al., 1977; Yu and Campagnoni, 1982; Agrawal et al., 1982). Studies on 14 kd and 18.5 kd forms from the rat have shown that these two proteins differ by only a 40 amino acid insertion, located immediately after the equivalent of residue 113 of mouse 14 kd MBP (Martenson et al., 1972; Dunkley and Carnegie, 1974). This position corresponds precisely to the breakpoint between the last and next to last exon encoding the mRNA for mouse 14 kd MBP. Since genomic blot data, and the structures of the genomic clones reported here are consistent with there being a single MBP gene in the mouse genome (see below), we sought to discover whether there might be, in the gene cloned in cos138, an exon which can encode this C-terminal insertion. A family of synthetic oligonucleotides of length 17 was synthesized using an automated instrument employing phosphoramidite triester chemistry (Hunkapiller et al., 1984). Reverse translation of amino acids 138 to 143 (His-Lys-Gly-Phe-Lys-Gly) of human 18.5 kd MBP allowed the synthesis of two pools of 32 sequences each (LMBP-1.17 CC(T/C)TT(A/G)AA(A/G)CC(T/C)TT(A/G)TG, and LMBP-2.17 CC(T/C)TT(AG)AA(T/C)CC(T/C)TT(A/G)TG). Each of these mixtures of oligonucleotides was 5'-labeled with $\gamma[^{32}P]$ ATP and T4 polynucleotide kinase and used in a hybridization with a clone blot of DNA from the region between exons IV and VI. LMBP-2.17 hybridized with the 7.0 kb HindIII fragment from cos138 while LMBP-1.17 did not under the stringency conditions used (data not shown). Further

hybridizations of digests of the 7.0 kb HindIII fragment with radioactively labeled LMBP-2.17 localized the hybridizing region to a 0.65 kb AvaII-SacI fragment. The nucleotide sequence of this fragment was determined and is presented in part in Figure 1. The position of the exon found is also shown in Figure 1, where it is labeled "V". The oligonucleotide hybridizing region, positions E64 to E80, would be predicted to hybridize with LMBP-2.17 and not LMBP-1.17 under stringent conditons. The 3' and 5' splice sequences are presented at nucleotides E-1 to E-12, and E124 to E129, respectively, and are in good agreement with the consensus sequences distilled by Mount (1982). Between these splice points lies an open reading frame of 123 bp which translates to 41 amino acids, differing from the human sequence at two positions. Residue 118, lysine in the mouse exon, is arginine in the human protein, and alanine-tyrosine, residues 140-141 of the mouse sequence are replaced by valine in the human protein. Therefore, the gene on cos138 has the ability to encode mouse 14 kd MBP if exon V is spliced out, and mouse 18.5 kd MBP if exon V is included in the processed transcript. Since there is no reason to propose more than one MBP gene in mouse (see below), it appears that this gene gives rise, through alternate splicing of primary transcripts, to distinct messenger RNAs encoding both the 14 kd and 18.5 kd MBPs.

There Do Not Appear to be Additional MBP Genes in the Mouse Genome

If the gene cloned on cos138 is the only MBP gene of the mouse, the restriction map of this clone, presented in Figure 1, and the flanking regions in the genome should predict the band patterns observed in Southern blots using a cloned cDNA probe. Digestion of mouse DNA with the restriction enzyme Hind III should produce MBP-hybridizing bands of lengths 3.2 kb, 3.6 kb and 7.0 kb, in addition to one additional band of greater than 4.5 kb. This last band would be expected to be the most intense since it contains exon VI, by far the largest of the MBP exons.

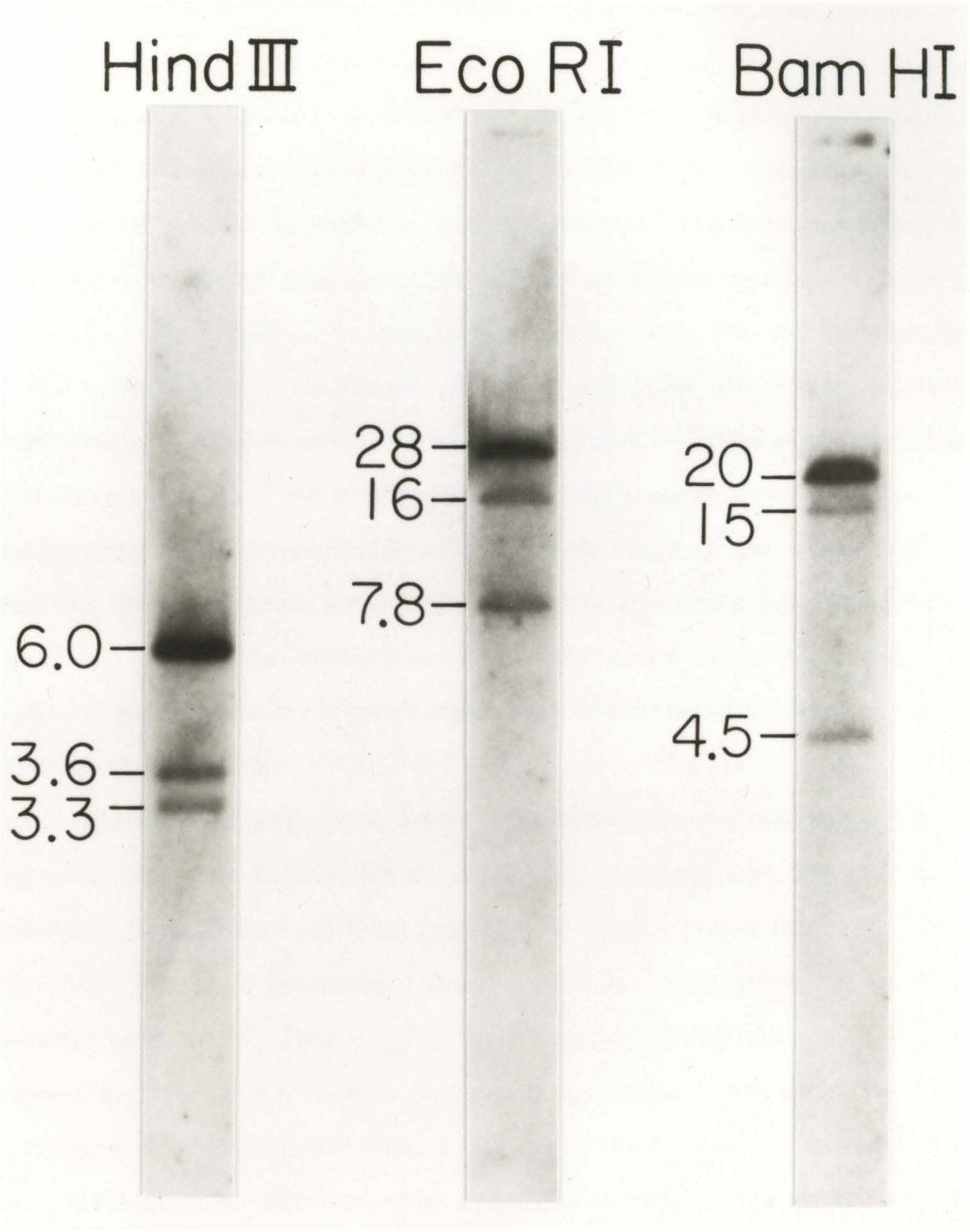
The restriction enzyme EcoRI would produce hybridizing fragments of 7.8 kb and 16 kb, and an additional, most intense band of greater than 7.4 kb. Digestion with the enzyme BamHI is expected to produce a hybridizing band of 4.5 kb, in addition to two bands of length greater than 11 kb, one of which would be the most intense.

Figure 2 shows a genomic Southern blot in which DNA from the SWV strain of mouse is digested with these three restriction enzymes and hybridized with the 1.5 kb rat cDNA insert from pMBP-1 (Chapter 1). This fragment contains material which hybridizes with 180 bp of mouse exon I, all of exons II, III and IV, and 1100 bp of exon VI. With the exception of the 7.0 kb HindIII band, the predictions made from the restriction map are all confirmed and there are no additional unexplained bands, indicating that there are not additional closely-related genes in the mouse genome. The 7.0 kb HindIII band will hybridize with the cDNA probe over only the 33 bp of exon IV. Although hybridization of this exon is detected on clone blots washed at lower stringency, this short region of homology is insufficient to produce a detectable signal at the high stringencies of hybridization and washing used in this genomic blotting experiment. The possibility of the existence of an additional gene or genes so closely related that all restriction sites are conserved is considered unlikely because Southern blot analysis with a cDNA probe involving 16 restriction sites, in several strains of mouse, failed to detect any polymorphisms.

The MBP Gene Produces mRNAs with a Single Major 5' End

The sequence presented as exon I encodes the N-terminal 56 amino acids of mouse MBP. The region from positions A42 to A218 shows a high degree of sequence homology with the 5' end of a cDNA clone for rat 14 kd MBP. In order to determine how much further in the 5' direction exon I extends, and whether this is the point at which transcription begins, S1 nuclease protection and primer

Figure 2. MBP hybridizing restriction fragments from SWV DNA. 3 μg of liver DNA from the SWV strain of mouse was digested with each of the restriction enzymes shown, electrophoresed through a 0.7% agarose gel, blotted onto nitrocellulose and hybridized with the nick-translated 1.5 kb EcoRI fragment from the rat cDNA clone pMBP-1 (Chapter 1). Hybridization was in 5X SSC at 68°C, and the filter was washed in 0.1 × SSC at 68°C.


Hind III
Eco RI
Bam HI
6.0
3.6
3.3
28
16
7.8
20
15
4.5

extension experiments were performed, as diagrammed in Figure 3A. The 220 bp PvuII-AvaII restriction fragment, 5'-labeled at the AvaII end, was gel-isolated, denatured and hybridized with poly(A) RNA from 18-20 day brain in 80% formamide at a temperature at which DNA-RNA hybrids would readily form but DNA-DNA hybrids would be unstable. The resulting hybrids were digested with single-strand specific S1 nuclease, and the protected fragments visualized by electrophoresis through denaturing gels followed by autoradiography, as shown in Figure 3B. The length of the protected fragment will indicate the distance between the 5' end of exon I and the AvaII site. For the primer extension experiment, the 78 bp AluI-AvaII restriction fragment, 5'-labeled at the AvaII end, was isolated and hybridized with poly(A) RNA from 18-20 day brains. Excess cold deoxynucleotides and reverse transcriptase were added to the solution of the resulting hybrids, allowing the primer fragment to be enzymatically extended to the 5' end of the annealed template RNA. The length to which the primer is extended will indicate the distance separating the position of the AvaII site from the 5' end of the mRNA.

The results of both the S1 protection and the primer extension experiment were run on a 5% polyacrylamide sequencing gel along with Maxam-Gilbert reactions of the 220 bp PvuII-AvaII fragment, as shown in Figure 3B.

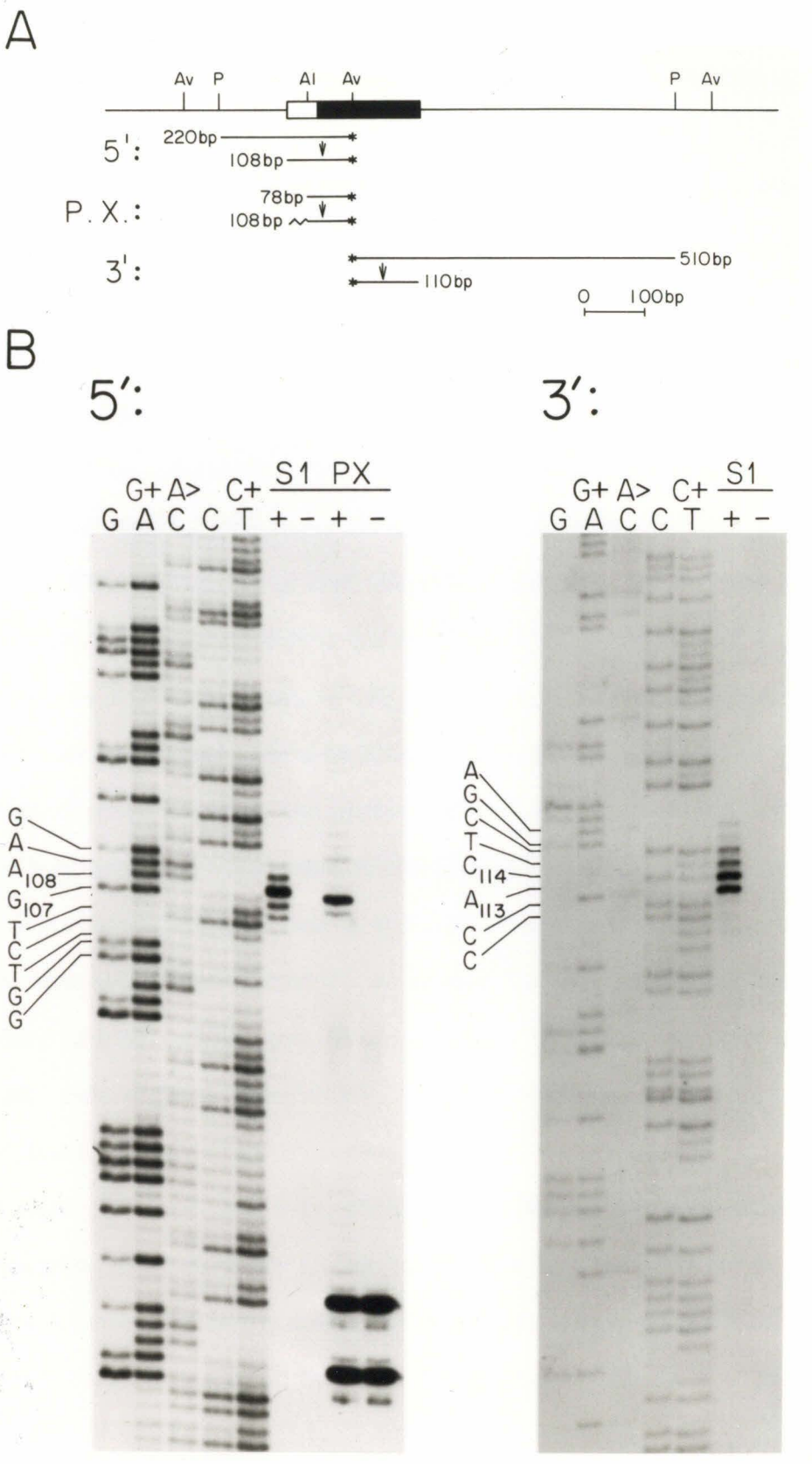
The major S1-protected fragment, lane S1+ from panel 5', migrates between positions G_{107} and A_{108} on the sequencing ladder, and is immediately flanked by three weaker bands. It is not clear whether these secondary bands correspond to minor initiation sites, or are due to the S1 nuclease leaving slightly heterogeneous ends. While the DNA fragments in both the sequencing reaction lanes and S1 protection lane have the same 5' ends, the piperidine cleavage step of the Maxam-Gilbert reactions yield 3' phosphate ends while S1 nuclease leaves 3' hydroxyl ends, preventing direct comparison of mobilities of fragments. In

Figure 3. Determination of the 5' and 3' boundaries of exon I. A. Filled box, protein-coding portion of exon I, open box, 5' untranslated region; single line, flanking sequences; A1, AluI; Av, AvaII; P, PvuII. The 220 bp, 5'-labeled probe fragment, the 510 bp 3'-labeled probe fragment and the 78 bp 5'-labeled primer fragment are shown. Small arrows represent the action of S1 nuclease on protected fragments (5' and 3') and the action of reverse transcriptase on the primer fragment in the primer extension (PX) experiment.

B5'. Autoradiogram of a 5% acrylamide 50% urea of gel through which the products of DNA squencing reactions on the 220 bp AvaII-PvuII fragment were run with the products of the 5' S1 and primer extension experiments. For the S1 experiment either 0.25 μg of poly(A) RNA from 18-20 day brain plus 19 μg of *Aplysia* carrier RNA (lane S1+) or 19 μg of *Aplysia* carrier RNA alone (lane S1-) was annealed with the 220 bp PvuII-AvaII fragment 5'-labeled at the AvaII end and incubated with S1 nuclease, as described in Experimental Methods. One-fourth of the products were loaded on the gel shown. For the primer extension experiment with 1.0 μg of 18-20 day brain RNA plus 9.5 μg of *Aplysia* carrier RNA (lane PX+) or 9.5 μg of carrier RNA alone (lane PX-) was annealed with the 78 bp AluI-AvaII primer fragment 5'-labeled at the AvaII end, and the primer extended with reverse transcriptase, as described in Experimental Procedures. One-twentieth of the resulting products were loaded on the gel shown. The nucleotides G_{107} and A_{108} are numbered from the 5'-labeled end of the sequencing fragment.

B3'. Autoradiogram of 5% acrylamide 50% urea gel through which the products of DNA sequencing reactions on the 510 bp AvaII-PvuII fragment were run with the products of the 3' S1 protection experiment. For the S1 experiment the AvaII-PvuII fragment, 3'-labeled at the AvaII site, was annealed with either 1 μg of 18-20 day brain RNA plus 19 μg liver poly(A)-carrier RNA (lane S1+), or with 19 μg of carrier RNA only (lane S1-), and incubated with S1 nuclease as

described in Experimental Procedures. One-sixth of the total products were run on this gel. The nucleotides A_{113} and C_{114} are numbered from the 3'-labeled end of the sequencing fragment.


A
Av
P
Al
Av
P
Av
5':
220bp
108bp
P. X.:
78bp
108bp
3':
510bp
110bp
0
100bp
B
5':
3':
G+ A> C+
S1 PX
G A C C T + - + -
S1
G A C C T + -
G
A
A108
G107
T
C
T
G
G
A
G
C
T
C114
A113
C
C

addition we do not know which of the several S1 stop points represents the last nucleotide complementary to the mRNA sequence. However, it is clear that exon I begins at or very near position A1 in the nucleotide sequence given in Figure 1.

The dominant product of the primer extension experiment (lane PX+ from panel 5') is a fragment which comigrates with G_{107} in the accompanying sequence ladder (Figure 3). The very close agreement between the length of this fragment and the major products of S1 protection indicates that MBP mRNA is initiated at the 5' end of exon I and that there is not another exon which encodes uniquely 5' untranslated material. The nucleotide at position A1 is part of a CA dinucleotide, at which many eukayotic RNA polymerase II transcripts are observed to initiate (Corden et al., 1980). Since the primer fragment used here comes from a region that would be common to the mRNAs for all four forms of MBP, and a single 5' end is indicated by the experiment, it can be concluded that a common 5' end is used for the messengers which are to be subsequently spliced differently.

The two intense bands at the bottom of both PX lanes correspond to the 78 bp primer fragment and a fragment of similar size, labeled on the other strand, which was co-purified. The presence of this band does not affect the conclusions drawn from this experiment since it is labeled on the strand which is not complementary to MBP mRNA, and the same primer extension end point has been found in an independent experiment using a different primer fragment (unpublished data).

Both lane S1+ and lane PX+ show a distribution of very weak bands extending upwards from the major strong bands, to a position 17 nt longer. These may represent a number of rarely used alternate initiation points for transcription but in any event constitute a small fraction of transcription of the MBP gene.

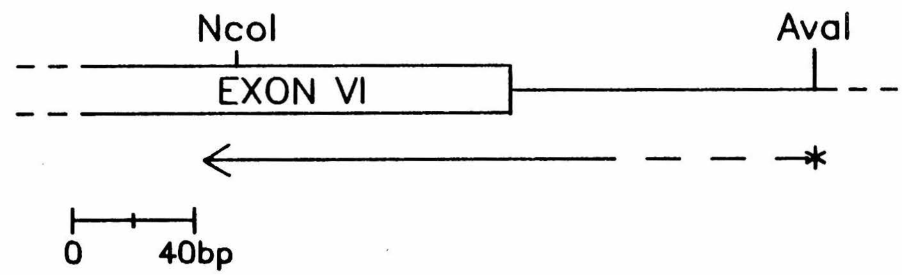
The 3' End of Exon I

Sequence homology between the rat cDNA clone for 14 kd MBP and exon I ends at position A219 in Figure 1. This nucleotide is part of a 5' splice sequence (positions A216-A224) which would indicate splicing after the G nucleotide at position A218. Beyond this site there is an in-frame termination codon at position A234. An S1 nuclease protection experiment was performed to determine that transcripts are actually spliced at the predicted position. The 510 bp AvaII-PvuII fragment, 3'-labeled at the AvaII end, was isolated and hybridized with poly(A) RNA from 18-20 day brains. After incubation with S1 nuclease the protected fragments were electrophoresed through a denaturing gel with Maxam-Gilbert reactions of the same DNA fragment. This experiment is presented in Figure 3. The RNA protected the probe fragment to lengths that comigrate with bands A_{113} and C_{114}, which correspond to positions A220 and A221 in Figure 1. When a 3'-labeled fragment is used in Maxam-Gilbert reactions, chemical modification of a base at position n will leave, after cleavage, an end-labeled fragment of length n-1 nt with 3' hydroxyl and 5' phosphate ends. Therefore the two major S1 bands correspond to protection to positions A219 and A220, and the S1 protection experiment results in protection to a position within one nucleotide of that predicted from nucleotide sequence, confirming the assignment of splice site made from comparison with the rat cDNA sequence.

Mouse and Rat mRNAs are Polyadenylated at Analogous Positions

The 3' end of exon VI has been assigned by homology with the sequence of the 3' end of the cDNA clone of the mRNA for rat 14 kd MBP. The several tandem AATAAA sequences found in the rat cDNA are also found in the mouse genomic sequence at positions 67 to 81 (Figure 4). Cleavage and polyadenylation would be expected to occur at position 87 or 88. An S1 protection experiment was

Figure 4. DNA sequence homology between the 3' end of mouse exon VI and the rat cDNA clone λRB7. Box represents the 3' end of mouse MBP exon VI (approximate map position from Figure 1 = 32 kb); single line, flanking DNA. Arrow indicates DNA sequence read from the 3'-labeled AvaI site. The DNA sequence from mouse is numbered from the first nucleotide of the NcoI site, underlined. The sequence from the 3' end of the rat cDNA clone (Chapter 1) is shown below, with dots indicating identity with the mouse sequence. The multiple tandem poly(A) addition sequence homologies are underlined at positions 67 to 81. The small vertical arrow marks the beginning of the poly(A) tail of the rat cDNA clone.




```
                   Nco I
MOUSE    -10   CGACCTTGCCCATGGCTTCGTCCAG C GCGCAGCTGCAGTATGGCTGGACAGAAGCACC    48
RAT            ................. .A.....A.A..A.............................
                        |        |        |        |        |

MOUSE     49   TACTATTCTTGAATATTGAAATAAAATAATAAACTTGCAATGATCTTTG               97
RAT            ....G......G.........................AA.AAAAAA
                                                    ↑
```

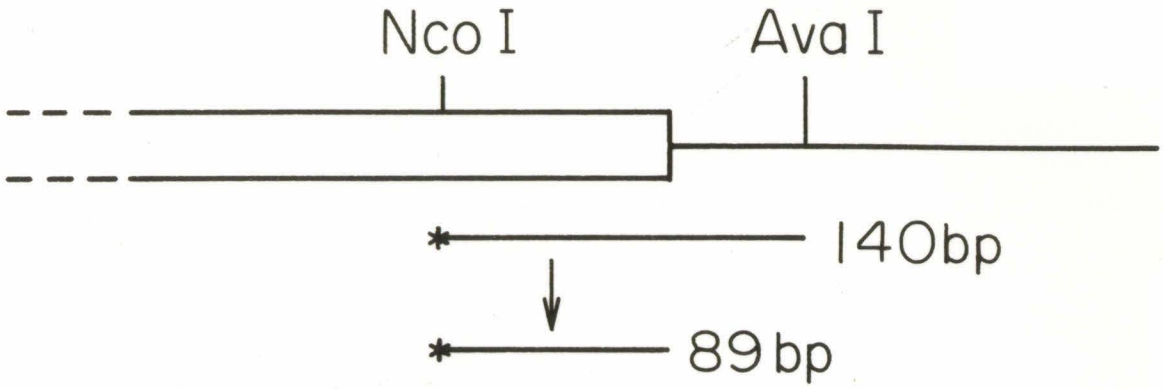
performed to determine whether this site does indeed represent the 3' end of exon VI. Figure 5 shows the 140 bp NcoI-AvaI fragment, 3' end-labeled at the NcoI end, which was isolated and hybridized with poly(A) RNA from 18-20 day brain. The resulting hybrids were incubated with S1 nuclease, and the protected fragments electrophoresed through a denaturing gel with end-labeled restriction fragments as size standards (Figure 5). The end-labeled probe fragment is protected to a length of 89 nt by brain RNA, indicating that the end of exon VI is very near position 89 in Figure 4, as predicted.

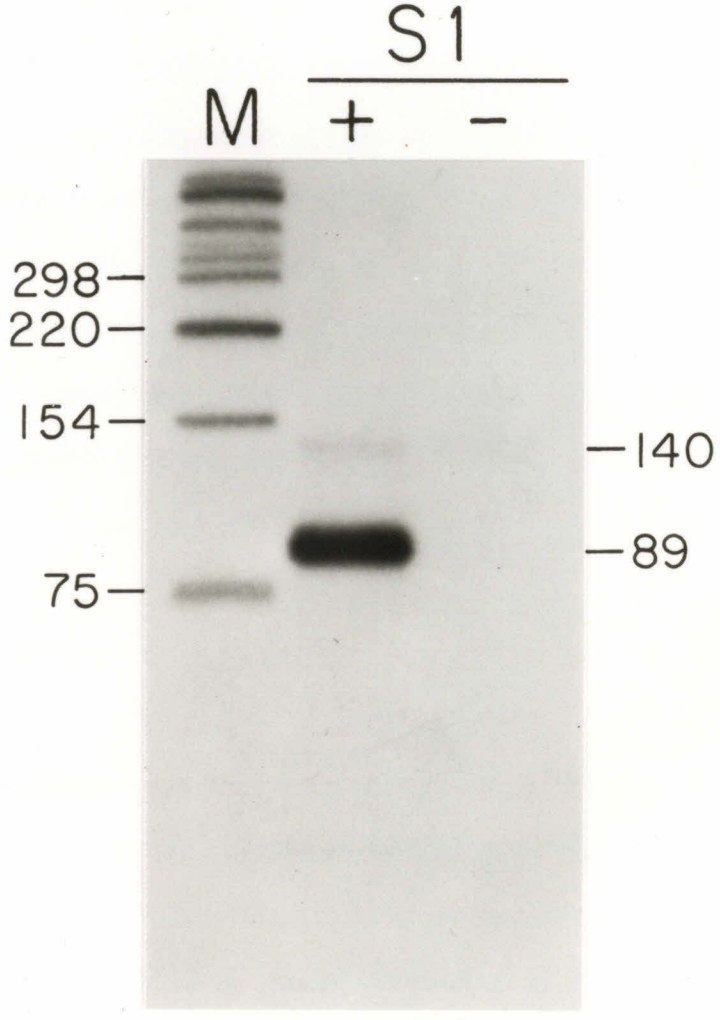
Although we do not have sequence data from mouse cDNA clones, several lines of evidence suggest that the region around position 89 in Figure 4 is also the site of polyadenylation. First, the high degree of sequence homology between the rat cDNA clone and the mouse exon indicate this is where polyadenylation would be expected to occur. Second, the S1 protection experiment presented in Figure 5 indicates that this is in fact the 3' end of exon VI. Third, there is no good 5' splice site near this position, but there is a repeated poly(A) addition signal just upstream. These observations strongly suggest that the exon ends not by virtue of a splicing event, but rather by cleavage and polyadenylation of the primary transcripts.

Is There a Seventh Exon in the Mouse MBP Gene?

The data presented here show that the MBP gene cloned on cos138 can encode both the 14 kd and the 18.5 kd MBPs. Mouse myelin also contains smaller amounts of 17 kd and 21.5 kd MBPs. In adult CNS myelin the four forms are present in relative amounts 1:10:3.5:35 for the 21.5 kd, 18.5 kd, 17 kd and 14 kd forms respectively (Barbarese et al., 1978). While the two minor forms differ from the 14 kd and 18.5 kd by about 30 additional amino acid residues somewhere in the N-terminal half of the protein (Barbarese et al., 1977), they are not metabolically-

Figure 5. Determination of the 3' end of exon VI. Box, 3' end of exon VI; single line, flanking DNA. The 140 bp NcoI-AvaI fragment, 3'-labeled at the NcoI end, was isolated and annealed with either 0.5 μg of 18-20 day brain RNA plus 9.5 μg of *Aplysia* carrier RNA (lane S1+), or with 9.5 μg carrier RNA alone (lane S1-) and incubated with S1 nuclease as described in Experimental Procedures. One-half of the resulting products were run on a 5% acrylamide, 50% urea gel with end-labeled DNA markers (lane M), and the gel dried and autoradiographed. Sizes are in nucleotides.


Nco I
Ava I
140bp
89 bp


S1
M
+
−
298
220
154
75
140
89

related precursors of the two major forms (Yu and Campagnoni, 1982). Recent peptide map data indicate that the sheep 21.5 kd MBP differs from the 18.5 kd form by an insertion of 30 amino acids of known composition, but unknown sequence, between residues lysine 56 and aspartic acid 57 (Carnegie and Dowse, 1984). This position corresponds precisely to the break between exons I and II of the mouse MBP gene described here. Since it is very likely that there is a single MBP gene in mouse, it is probable that there is a seventh exon encoding these additional amino acids located between map positions 0.2 and 18.8 of Figure 1.

Several attempts to detect the proposed exon have been unsuccessful. These include making radioactive cDNA with a primer specific for MBP exon II, and 18-20 day mouse brain RNA as template, for use as a probe of DNA blots of restriction fragments from subclones of the MBP gene, and nick-translation of restriction fragments from across the 18.6 kb region for use as hybridization probes of RNA blots. The short length of the proposed exon (90 bp) and its relatively rare expression combine to make its detection difficult. The application of new approaches to this problem should definitively resolve the question of the existence and location of the DNA encoding the extra 30 amino acids of the two minor forms of MBP.

Discussion

The preceding data demonstrate that mice have a single 32 kb gene containing six exons which can give rise to the mRNAs for either the 14 kd or the 18.5 kd MBP, by alternate patterns of RNA splicing. It has been determined that transcription initiates 47 ± 1 bp 5' of the AUG initiation codon used in MBP synthesis. The 5' flanking region contains a weak homology to the TATA box consensus (Breathnach and Chambon, 1981) at positions A-33 to A-28, TTCAAA. The sequence CCACT,

at position A-84, is the best match for the CCAAT box sequence, expected in the -70 to -95 bp region (Efstradiatis et al., 1980).

There are 47 bp of 5' untranslated material prior to the initiator AUG codon (position A48), which immediately precedes the codon for alanine, the amino terminal residue of mature MBP. Five bp prior to the initiator AUG there is a second AUG triplet, at position A43. This triplet is not functional since it is immediately followed in frame by the termination codon TGA.

All exons are flanked by the expected splice sequences, and splicing always occurs between codons. The long 3' untranslated region is largely conserved relative to the rat, and cleavage and polyadenylation of the mouse mRNA occurs at the site corresponding to that identified by sequence analysis of a rat cDNA clone.

The synthesis of multiple mRNAs from a single cellular gene, to produce distinct protein species, has been reported in several systems. In some of these cases the different proteins have known functional or developmental specificities. The choice between mRNAs for the membrane and secreted forms of immunoglobulin μ chains is determined by the poly(A) addition site used, which either retains or excludes two exons from the polyadenylated, unspliced precursor (Early et al., 1980). The expression of chicken myosin light chains LC_1 and LC_3 is developmentally regulated. They are translated from two mRNAs which are produced from the same gene, but differ as the result of alternate transcription initiation and splicing patterns (Nabeshima et al., 1984). The mRNA for calcitonin, and calcitonin gene-related product RNA are generated by use of either one of two exons, both of which are apparently present in the same precursor (Amara et al., 1982). The expression of these two RNAs changes dramatically in serially transplanted rat medullary carcinoma lines. The αA-crystallin gene in mouse generates two distinct mRNAs for αA- and αA^{ins}-

crystallins by the inclusion or exclusion of a 69 bp exon in the processed transcript (King and Piatigorsky, 1983). No functional or developmental specificities for the αA and αA^{ins} forms are known.

The alternate splicing pathways of the MBP gene are similar to those of the αA-crystallin gene in that the optional information is contained in an exon situated between two others which are always included in the processed transcript. The reasons for the frequent removal of these optional exons during splicing are not known. The 5' splice site immediately following the optionally-used second exon of the αA-crystallin gene departs from the GT/AG rule (Breathnach and Chambon, 1981) but otherwise is a good match for the consensus sequence of Mount (1982). The 3' splice site of the intron preceding the second exon also departs from the consensus in that the fifth nucleotide 5' of the splice site, a pyrimidine in 111 out of 130 sequences compared by Mount, is A in the αA-crystallin sequence. In contrast, the splice sequences flanking exon V of the mouse MBP gene do not depart from the GT/AG rule, and are generally in agreement with the published consensus sequences.

It is very probable that there is a second, less-frequently used exon lying in what is here designated as the first intron, encoding the 30 amino acids which distinguish 21.5 kd and 17 kd MBPs from the 18.5 kd and 14 kd forms. The molar ratios of the four forms, 1:10:3.5:35 in order of decreasing size (Barbarese et al., 1978) are consistent with frequencies of use of 28% for exon V and 10% for the proposed exon encoding the extra 30 amino acids.

The existence of four MBPs in mouse raises questions concerning the roles of the four forms. The relative amounts of the four proteins reported were derived from analysis of total brain myelin. It could be the case that regions of the nervous system or classes of oligodendrocytes and Schwann cells express different relative amounts which, when averaged over the whole brain, result in

the ratios reported. Minor changes in the relative amounts of the four forms are reported to occur during myelin accumulation in young mice, but no functional significance has been proposed for this difference (Carson et al., 1983). While compelling evidence does not exist, the function of the MBPs in myelin is thought to relate to the maintenance of proper structure, possibly by causing the cytoplasmic faces of adjacent membranes to lie in close aposition without the presence of apreciable cytoplasm. It has been proposed that the interspersion of basic and hydrophobic residues in the MBPs allows the protein chain to lie along the surface of the membrane and interact with acidic head groups of phospholipids and other, hydrophobic regions. The amino acid sequence encoded by exon V and the amino acid composition of the N-terminal insertion (Carnegie and Dowse, 1984) are similar to the rest of the MBP regions and suggest that these portions of the proteins could also play such a role. It may be relevant to note that several species including human and bovine, construct functional if sometimes clumsy nervous systems with only the 18.5 kd MBP present in detectable amounts.

The cloning and characterization of the normal mouse MBP gene presented here allows several new lines of inquiry. Cloned DNA probes can be applied to the question of region and cell-type specific expression of multiple forms of MBP. The normal gene can be used as a standard for the detailed molecular analysis of MBP expression in the shiverer mutant mouse (Chapter 3). A conclusive test of the hypothesis that the MBP deletion is the shiverer lesion, is feasible in the form of microinjection of mutant embryos with the cloned wild-type gene. The cloned gene may also be used in studies of the requirements for expression of a nervous system-specific gene. Because of the interesting splicing pattern found, this gene may prove useful in studies examining mechanisms and requirements for RNA splicing.

Experimental Methods

Library construction

Liver DNA from the SWV strain of mouse and was partially digested with Sau3AI and cloned into the cosmid vector pTL5 (Lund et al., 1982) as described by Steinmetz et al. (1982). 5×10^5 colonies were screened with a cloned rat cDNA probe (Chapter 1). Six hybridizing clones were isolated and two were characterized in detail. Restriction maps of clones cos13 and cos138 are shown in Figure 1.

DNA sequencing

Restriction fragments from cos138 were subcloned into plasmid vectors pUC9, pUC13 and pBR325. Restriction fragments which hybridized with the rat cDNA clone were sequenced by the method of Maxam and Gilbert (1980).

Detection of exon V

The amino acid sequence for residues 139 to 144 of human MBP (Carnegie, 1971) was reverse translated, giving a mixture of 64 sequences of length 17.

Protein:	His	Lys	Gly	Phe	Lys	Gly
RNA:	5' CA(U/C)	AA(A/G)	GGN	UU(A/G)	AA(A/G)	GGN 3'
LMBP-1.17:	5' CC(T/C)	TT(A/G)	AA(A/G)	CC(T/C)	TT(A/G)	TG 3'
LMBP-2.17:	5' CC(T/C)	TT(A/G)	AA(C/T)	CC(T/C)	TT(A/G)	TG 3'

The two mixtures of 32 sequences each were synthesized using an automated DNA synthesizer (Applied Biosystems) and 5'-labeled with T4 kinase and $\gamma[^{32}P]$ATP as described (Chapter 1). Clone blots of subclones of the MBP gene were prehybridized and hybridized at 42°C as described in Chapter 1, using 10^6 cpm/ml of labeled oligonucleotides in the hybridization.

Genetic blots

DNA was prepared from mouse livers by the method of Blin and Stafford (1976). After restriction digestion, partial depurination (Wahl et al., 1979) and blotting by the method of Southern (1975), nitrocellulose filters were prehybridized and hybridized (Maniatis et al., 1982) with nick-translated (Rigby et al., 1977) restriction fragments.

S1 nuclease protection experiments

Restriction fragments were prepared, end-labeled at one 5' or 3' end as indicated in figure legends, as described by Maxam and Gilbert (1980). The strand separation temperature of each double-stranded fragment was determined by heating aliquots of 80% formamide hybridization buffer (Favaloro et al., 1980) containing the fragment to increasing temperatures, quickly diluting and freezing the heated samples, followed by electrophoresis through non-denaturing acrylamide gels and autoradiography. An abrupt change in mobility of the fragment indicated that strand separation had occurred. Optimal S1 nuclease conditions were established by preparing aliquots of composition identical to that of annealed mixtures to be digested, containing either double-stranded 3'-labeled probe fragment or strand separated probe fragment. Aliquots were incubated with increasing S1 nuclease concentrations and the products visualized by autoradiography of denaturing gels. Annealing of probe fragments with RNAs and S1 nuclease digestion of the resulting hybrids were performed as described by Favaloro et al. (1980).

Primer Extension

The 78 bp AvaII-AluI fragment was 5' end-labeled and isolated as described by Maxam and Gilbert (1980). The strand separation temperature of the fragment

was determined, and it was annealed with RNA as described above. The mixture was ethanol precipitated, redissolved in 100 mM Tris pH 8.3, 10 mM $MgCl_2$, 70 mM KCl, 30 mM 2-mercaptoethanol, and dATP, dCTP, dGTP and dTTP were added to 0.2 mM. Five units of AMV reverse transcriptase (Life Sciences) were added and the mixture incubated at 42°C for 1 hour. The resulting mixture was denatured by heating to 90°C with 4 volumes of 90% formamide, 10 mM NaOH before loading on denaturing acrylamide gels.

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Chapter 3

A Molecular Analysis of the Shiverer Mutant Mouse: The Myelin Basic Protein Gene is Linked to the Shiverer Locus

and

Characterization of the Partially Deleted Myelin Basic Protein Gene, and its Transcription, in Shiverer Mice

Introduction

Mutations which disrupt developmental processes offer the potential for revealing insights into the events of normal development. The shiverer mutation is one of several which disrupt myelination and consequently neurologic function in the mouse. While many myelination mutations have been characterized in terms of genetics, anatomy, neurophysiology, biochemistry and pathology, for nearly all the nature of the primary lesion remains obscure (Baumann, 1980). Shiverer homozygotes appear unaffected until about 14 days after birth, the time of onset of major myelination of the central nervous system (CNS). The phenotype then consists of neurologic dysfunction of increasing severity, beginning with occasional shivers when the animal is active or disturbed, and progressing to tonic seizures and premature death during an attack (Chernoff, 1981).

In addition to a general reduction in amount of central myelin, a feature common to some other myelination mutants, central nervous system (CNS) myelin in shiverers has a specific ultrastructural defect. The major dense line, a prominent electron micrographic feature representing the area of contact of the cytoplasmic faces of the wrapped myelin membrane (Robertson, 1955) is largely absent in shiverer myelin and a thin layer of oligodendrocyte cytoplasm is retained in the wrappings (Privat et al., 1979; Rosenbluth, 1980a). Myelin basic protein (MBP), which is one of the major protein species in myelin (for review, see Carnegie and Moore, 1980), has been shown to be closely associated with the major dense line (Omlin et al., 1982). The most striking biochemical defect in shiverer mice is the absence of MBP. Published reports differ on whether or not there are very low amounts of MBP antigens in CNS myelin of *shi/shi* animals, but in any case the level is agreed to be less than 0.1% of +/+ levels (Dupouey et al., 1979; Ganser and Kirschner, 1980; Lachapelle et al., 1980). Radioimmune assay detects 0.4% of

+/+ levels of MBP in peripheral nervous system (PNS) myelin (Ganser and Kirschner, 1980). Campagnoni et al. (1984) demonstrated that *shi/shi* polysomes incorporate no detectable [^{35}S] methionine into immunoprecipitable MBP, while *shi*/+ polysomes incorporate half the +/+ value.

Since myelin is the result of a close interaction between the myelinating glial cell and an axon, it is relevant to inquire whether the primary site of action of a myelination mutation is the glial cell, or whether it might act in the neuron to disrupt some signal essential for normal myelination. Co-culturing of neurons and glial cells from the CNS (Billings-Gagliardi et al., 1984) and peripheral nervous system (PNS) (Shine and Sidman, 1984) of wild type and shiverer mice, and immunocytochemical examination of PNS myelin of shiverer-normal chimeric mice (Peterson and Marler, 1983) all indicate that the shiverer lesion acts directly on oligodendrocytes and Schwann cells.

In contrast to the disastrous phenotype of the mutation in central myelin, PNS myelin of shiverer mice has a nearly normal morphology, indicating that MBP is not essential for myelination by Schwann cells (Kirschner and Ganser, 1980; Rosenbluth, 1980b). Possible reasons for this are discussed below.

It has previously been shown, using a cloned cDNA for MBP, that the brains of *shi/shi* mice contain greatly reduced amounts of MBP mRNA, and that some but not all of their MBP gene is deleted (Chapter 1).

The findings summarized above lead to three questions. What is the nature and extent of the deletion of MBP sequences in *shi/shi* mice? Can there be any *bona fide* MBP antigens synthesized by mice carrying this deletion? Is the partial deletion of the MBP gene the shiverer locus? As a result of the cloning and characterization of the wild type MBP gene, presented in Chapter 2, it is possible to apply molecular approaches to these problems. In the present work the MBP deletion is characterized and found to remove all but the first exon of five

required for MBP synthesis. It is shown that a reduced level of MBP RNA is present in shiverer brains, and that it is incorrectly processed. This RNA, if translated, could encode the first 56 amino acids of MBP. The MBP gene is mapped to mouse chromosome 18, consistent with genetic mapping of the shiverer locus.

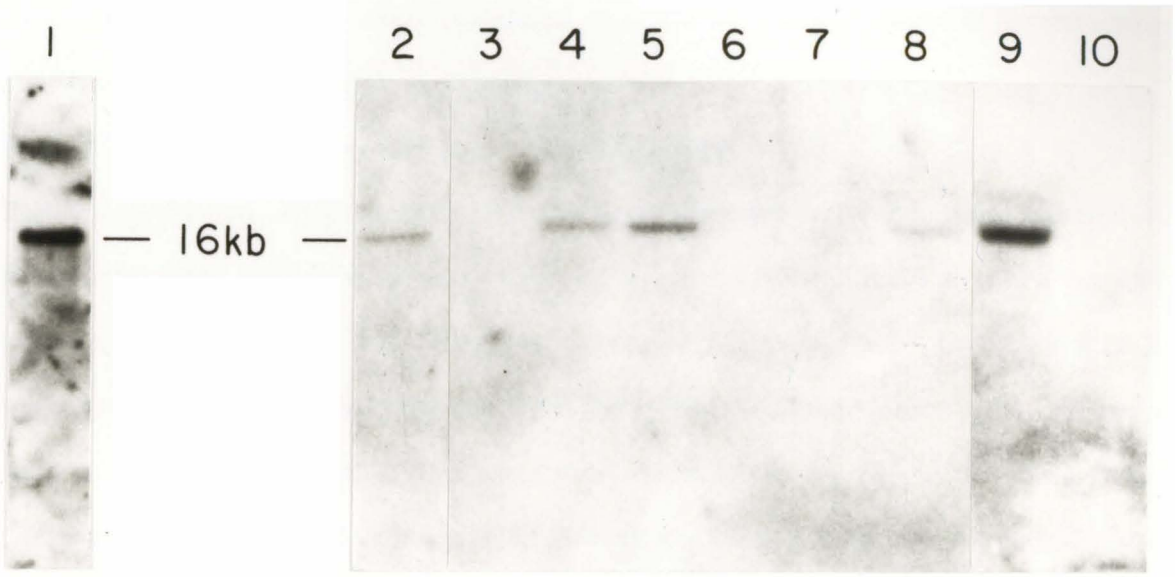
Results

The Mouse MBP Gene and the Shiverer Mutation are Both on Chromosome 18

The shiverer mutation has been mapped to murine chromosome 18 by Richard Sidman of Harvard University (personal communication) by classical genetics. Previous work has shown that shiverer mice have deleted part of their MBP genes (Chapter 1). A lesion in the MBP gene can explain both the observations of lack of MBP and loss of the major dense line in CNS myelin, while other phenotypes associated with the mutation could result from secondary effects of the absence of MBP. If the shiverer mutation is a partial deletion of the MBP gene, it should be possible to show that the MBP gene is located on chromosome 18.

DNAs were prepared from a panel of Chinese hamster-mouse hybrid cell lines which contain different subsets of the mouse's chromosomal complement. DNAs from the hybrid cell lines were kindly made available by Dimitrina Pravtcheva and Frank Ruddle of Yale University. The DNAs were restriction digested, electrophoresed through agarose gels and blotted onto nitrocellulose. Hybridization with a radioactive probe taken from the cloned mouse MBP gene (Chapter 2) indicated which cell lines carried the mouse MBP gene. Figure 1 shows the result of hybridizing the nick-translated 3.8 kb BamHI fragment from the first intron of the MBP gene (Figure 2) with EcoRI-digested DNAs. Under the stringency conditions used no detectable hybridization occurs

Figure 1. Hybridization of DNAs from Hybrid Cell Lines with an MBP Probe. Approximately 5 μg of DNA isolated from each cell line was restriction digested with Eco RI, electrophoresed through a 0.8% agarose gel, and transferred to nitrocellulose as described in experimental procedures. Filters were hybridized with the nick-translated 3.8 kb BamHI-SalI fragment from MBP intron I (positions 14.5 to 18.3 in Figure 2), washed at high stringency, and autoradiographed. DNAs on gels were: lane 1, MACH 4A63; 2, TUCE 12G/4; 3, MFE 2/1/7; 4, MFE 2/3; 5, R44; 6, ECm4e; 7, MACH 4B31AZ3; 8, MFE 2/1/1; 9, BALB/c; 10, hamster cell line E36. All lanes except lane 1 are from the same gel. Appropriate positive and negative controls were present on the gel from which lane 1 is taken, but are not shown.


1
2
3
4
5
6
7
8
9
10
16kb

with Chinese hamster DNA (lane 10). The probe hybridizes with the expected 16 kb EcoRI fragment (see Figure 2 for restriction map) in mouse DNA (lane 9).

Table 1 presents the murine chromosomal complements of the hybrid cell lines examined, and summarizes the hybridization data. The eight cell lines tested provide at least two hybridization scores inconsistent with localization of the mouse MBP gene to any chromosome other than 18, for which no discordancies exist. It is therefore strongly indicated that the mouse MBP gene is on the same chromosome as the shiverer mutation.

Five of Six MBP Exons are Deleted in Shiverer DNA

Previous work has demonstrated that shiverer mice are lacking a major portion of their MBP gene, and have greatly reduced levels of MBP mRNA relative to wild type mice (Chapter 1). The molecular cloning of the mouse MBP gene (Chapter 2) makes possible a more detailed study of the MBP lesion in shiverer mice. In order to determine the nature of the structural defect, cloned restriction fragments from across the MBP gene were used as hybridization probes against restriction-digested genomic DNAs from both *shi/shi* and +/+ mice.

Figure 2 shows the structural map of the wild type MBP gene and identifies the probe fragments used in the experiments described below. In the first experiment, fragments 1, 2 and 3 were used as hybridization probes against both +/+ and *shi/shi* DNAs digested with HindIII. Figure 2 shows that probe 1, which consists of a 3.6 kb HindIII fragment carrying MBP exon I, hybridizes strongly with the expected 3.6 kb HindIII fragment in both +/+ and *shi/shi* DNA. It therefore is clear that the deletion does not remove this part of the gene. The weak band of 9.0 kb in lane 1 represents a repeated element found in the DNA flanking exon I on the probe fragment, since it does not appear when a cDNA probe with exon I but not the flanking regions is used to probe a HindIII digest (data not shown). Probe 2 is a 1.4 kb BamHI fragment from the first intron, and hybridized with the

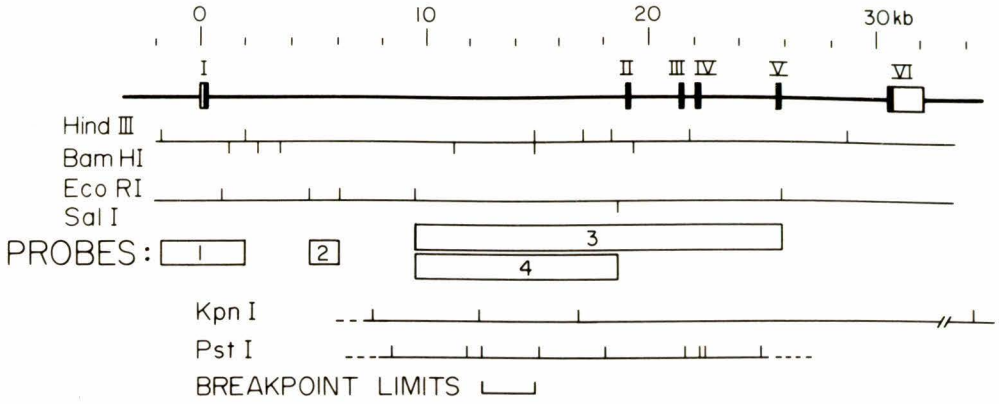
Table 1. Chromosomal Mapping of Mouse MBP Gene

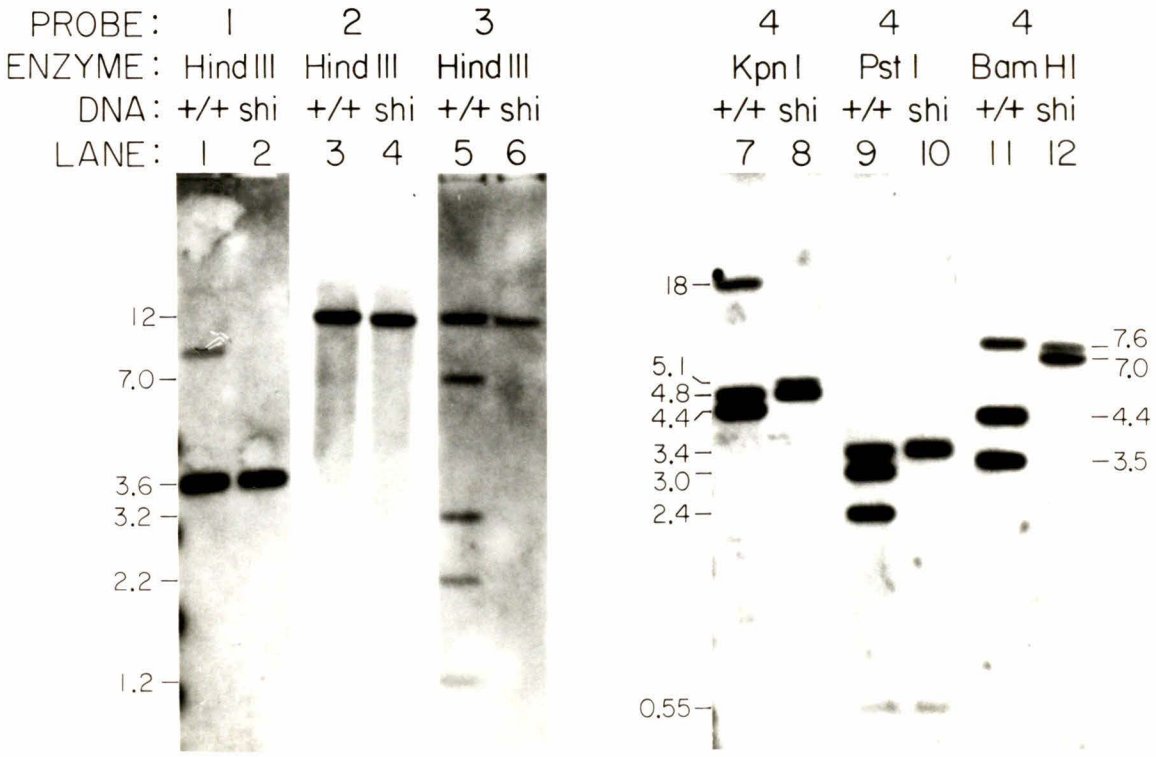
Hybrid Cell line	Mouse Chromosomes																					MBP Hybridization
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X	Y	
TUCE 12G/4	-	-	-	-	+	R	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-	+
MFE 2/3	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+
R44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+
MFE 2/1/2	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+
ECm 4e	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
MACH 4A63	-	+	-	R	-	-	+	R	-	-	-	R	+	-	+	+	-	+	+	-	-	+
MFE 2/1/7	+	+	+	-	-	+	+	+	+	-	-	+	+	-	+	-	+	-	+	+	-	-
MACH 4B31AZ3	-	+	-	-	-	-	+	-	-	-	-	+	-	-	+	+	-	-	+	-	-	-
Number of discordancies	5	4	4	2	4	2	4	3	5	2	4	3	2	4	4	4	2	0	3	3	5	

Symbols: +, present; -, not present; R, rearranged. Rearranged chromosomes were not included in the scoring. MBP hybridization scoring is taken from the data in Figure 1.

Figure 2. Determination of left breakpoint of the MBP deletion of shiverer mice. Upper part. Heavy line, structural map of wild type MBP gene cloned on cos148 (see Chapter 2). Scale is in kilobases from the 5' end of exon I. Restriction sites for six enzymes are given on the light lines, below. Boxes numbered 1-4 represent the restriction fragments used as hybridization probes in the experiment presented in the lower part. Breakpoint limits indicate the region within which the left breakpoint is determined to lie.

Lower Part. 9 μg of DNA from SWV (+/+) or homozygous shiverer (shi) mice was digested with the restriction enzymes indicated, electrophoresed and blotted. Filters were hybridized with the nick-translated probe fragments, as shown in upper part. Fragment sizes are in kilobase pairs.


0
10
20
30kb
I
II
III IV
V
VI
Hind III
Bam HI
Eco RI
Sal I
PROBES:
1
2
3
4
Kpn I
Pst I
BREAKPOINT LIMITS


PROBE: 1 2 3 4 4 4
ENZYME: Hind III Hind III Hind III Kpn I Pst I Bam HI
DNA: +/+ shi +/+ shi +/+ shi +/+ shi +/+ shi +/+ shi
LANE: 1 2 3 4 5 6 7 8 9 10 11 12
12
7.0
3.6
3.2
2.2
1.2
18
5.1
4.8
4.4
3.4
3.0
2.4
0.55
7.6
7.0
4.4
3.5

expected 12 kb HindIII fragment in +/+ DNA. The hybridizing fragment in *shi/shi* DNA migrates very slightly faster than that in +/+ DNA, but yields a signal of equal intensity. Probe 3 is a 16 kb EcoRI fragment from the middle of the gene carrying exons II, III, IV and V. It hybridizes with all the expected HindIII fragments from +/+ DNA, of lengths 12, 2.2, 1.2, 3.4 and 7.0 kb, listed from 5' to 3'. However, from *shi/shi* DNA a single HindIII fragment, of 11.8 kb, hybridizes with this probe. The autoradiographic signal is also reduced somewhat in intensity. The simplest interpretation of these patterns is that *shi/shi* mice carry a deletion which removes everything 3' of some position between the left end of the 16 kb EcoRI fragment and the right end of the 12 kb HindIII fragment. New sequences which now occupy the position adjacent to the remaining portion of the MBP gene would have a HindIII site at the position which would create a new, 11.8 kb HindIII junction fragment.

To increase the resolution of the mapping of the deletion breakpoint, restriction sites for additional enzymes were determined in the suspect region, by the method of Smith and Birnsteil (1976). Probe 4, shown in Figure 2, was used in hybridizations with digests of +/+ and *shi/shi* DNAs digested with some of these enzymes. For description of the results obtained with probe 4, restriction site positions are given in kilobases with reference to the 5' end of exon I. The HindIII site, 14.7 kb 3' of this reference point, is at position 14.7. A scale showing this coordinate system is at the top of Figure 2.

The KpnI fragments in +/+ DNA which hybridize with probe 4 are of sizes 4.8, 4.4 and 18 kb, listed from 5' to 3'. In *shi/shi* DNA the 18 and 4.4 kb fragments are missing, and a new weakly hybridizing junction fragment of 5.1 kb appears in addition to the 4.8 kb fragment. Therefore, the deletion spares the KpnI site at position 12.5 but removes the one at position 16.2. The low intensity due to the 5.1 kb junction fragment indicates that the breakpoint is near the site at 12.5.

BamHI fragments of sizes 7.6, 3.5 and 4.4 kb from +/+ DNA hybridize with probe 4, while in *shi/shi* DNA the two smallest fragments are missing and a new junction fragment of 7.0 kb appears. This indicates that the BamHI site at position 14.7 is lost in the deletion, but, as expected, the site at 11.3 is retained.

Probe 4 hybridizes with four PstI fragments from +/+ DNA, of sizes 3.4, 0.55, 2.4 and 3.0 kb. The small amount of hybridization expected between the 3' end of probe 4 and a 3.5 kb PstI fragment does not produce an autoradiographic band which can be resolved from the signal from the 3.4 kb fragment. In *shi/shi* DNA only the 3.4 and 0.55 kb fragments are detected, indicating that the PstI sites at 11.9 and 12.6 are conserved in the mutant genome. The failure to detect a new junction fragment indicates that either the PstI junction fragment is of 3.4 or 0.55 kb in size, or that the deletion breakpoint is very near the PstI site at 12.6 and produces a junction fragment which does not hybridize significantly with probe 4.

The preceding data localize the 5' end of the deletion to a position between the PstI site at 12.6 and the BamHI site at 14.7, probably nearer the PstI site. This region is indicated as "Breakpoint Limits" in Figure 2. While the size of the deletion, and its 3' end, are not known, it extends to at least the last exon of the MBP gene, 20 kb downstream. Previously published data indicate the loss of exon VI in *shi/shi* DNA. The 28 kb EcoRI and the 20 kb HindIII fragments from normal mouse DNA which hybridize with a rat cDNA probe, are now known to carry only exon VI (Chapter 2). Both of these fragments were shown to be missing from *shi/shi* DNA (Chapter 1). This result has been repeated numerous times. It therefore is concluded that the shiverer mouse suffers from a deletion which begins in intron I of the MBP gene and removes all MBP exons 3' of this point. The size and other breakpoint of the deletion are not known.

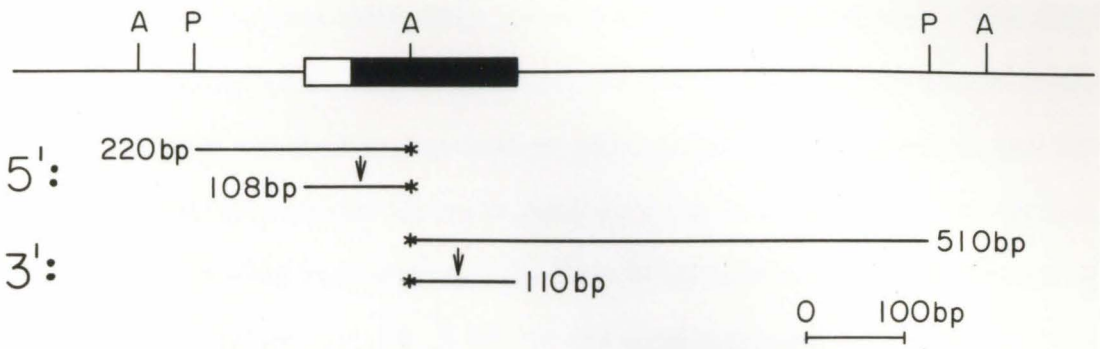
Correct Initiation of Transcription but Not Splicing in Shiverer Mice

While the MBP gene of shiverer mice is incomplete and therefore cannot encode a normal MBP mRNA, the above data argue that the first exon of the gene is present and could conceivably give rise to a transcript which might be translated to produce an MBP-related peptide. Signals for the expression of many mRNA-producing genes have been shown to be at the 5' end of the transcription unit (McKnight and Kingsbury, 1982; Dierks et al., 1983), and hence the transcription promoting elements of the MBP gene may have been spared by the shiverer deletion.

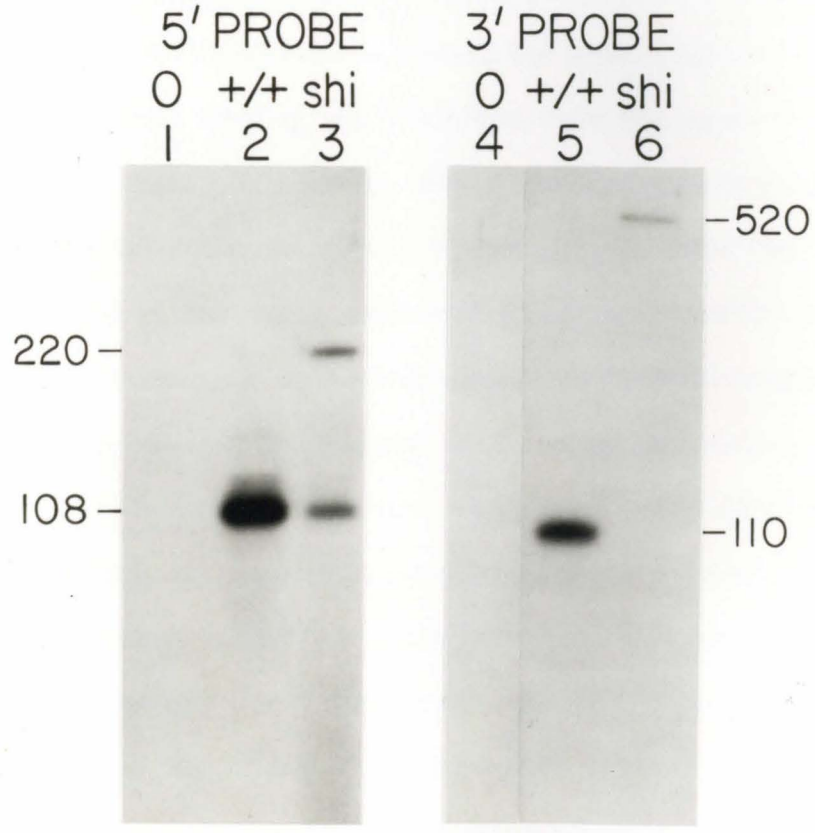
Previous work has shown that the steady-state level of MBP mRNA, as detected by a nick-translated rat cDNA clone in RNA blotting experiments, is reduced by greater than 100-fold in the brains of adult shiverer mice (Chapter 1). From knowledge of the structure of the mutant gene it was possible to design an assay for MBP gene transcription more sensitive than RNA blotting. An S1 nuclease protection experiment directed towards detecting the 5' end of MBP transcripts has the advantage that any RNAs which begin near the correct 5' end will be detected regardless of whether they are complete or are subsequently processed correctly. The experiment is also more sensitive and quantitative than RNA blotting. It does not, however, provide any information about the transcript structure 3' of the site of hybridization of the probe fragment. By performing in addition a separate S1 protection experiment to detect RNA spliced correctly at the 3' end of exon I it can be determined whether transcripts observed in the first experiment to initiate correctly are spliced correctly at the expected position at the 3' end of exon I splice point. S1 protection experiments were performed using brain RNA from 18-20 day shiverer mice, and the results obtained were compared with those from 18-20 day wild type mice. Figure 3A outlines the strategy used in these experiments.

Figure 3. S1 nuclease protection experiments with RNA from normal and shiverer mice. A. Upper line shows structural map of exon I and flanking sequences from the wild type (+/+) MBP gene. Below, lines indicate end-labeled DNA fragments isolated (longer lines) and protected (shorter lines) by +/+ RNA. Arrows represent action of S1 nuclease on protected fragments. Asterisks indicate that fragments were labeled at the AvaII ends. B. 10 μg of carrier yeast tRNA (O), 5 μg of carrier RNA and 5 μg of total brain RNA from +/+ mice (+/+), or 20 μg of total brain RNA from homozygous shiverer mice (shi), was annealed with the 220 bp AvaII-PvuII fragment (lanes 1-3) or the 510 bp AvaII-PvuII fragment (lanes 4-6), digested with S1 nuclease, and the resulting mixture electrophoresed through a 5% acrylamide, 50% urea gel. After drying, the gel was autoradiographed and is presented here. Sizes are in nucleotides (determined from end-labeled DNA size markers). The size marker which reads "520" should read "510".

A


A
P
A
P
A
5':
220bp
108bp
3':
510bp
110bp
0
100bp

B


5′ PROBE
3′ PROBE
0
+/+
shi
0
+/+
shi
1
2
3
4
5
6
220
108
520
110

For analysis of the 5' end, a 0.22 kb PvuII-AvaII fragment, 5'-labeled with ^{32}P at the AvaII end only, was labeled and isolated from a subclone of exon I and its flanking regions. The labeled end is from the AvaII site 108 bp from the 5' end of exon I. This fragment was hybridized with RNA in 80% formamide at a temperature chosen so that DNA-DNA hybrids would be unstable while DNA-RNA hybrids would readily form. Digestion of the resulting mixture with single-strand specific S1 nuclease resulted in digestion of unannealed probe fragments, and left labeled fragments of reduced discrete size deriving from annealed molecules. Analysis of the resulting radioactive fragments by autoradiography of denaturing gels indicates the distance of the 5' end of the exon from the AvaII labeling site, and the relative abundance of transcripts initiating there.

Lanes 2 and 3 of Figure 3B show the results of protection with 5 μg of total brain RNA from +/+ mice, and 20 μg of total brain RNA from *shi/shi* mice, respectively. RNA from normal mice protects the probe fragment for a distance of 108 nt from the AvaII labeling site. Previous work has shown this to indicate the correct 5' end of exon I (Chapter 2). RNA from the brains of *shi/shi* mice also protects the probe fragment to 108 nt. It therefore is clear that although no mRNAs are detected in RNA blots, some level of correct initiation and extension of MBP transcripts does occur in shiverer mice. Gel slices corresponding to the 108 nt bands were solubilized and counted to determine the relative abundance of MBP transcripts in +/+ and *shi/shi* brains. 5 μg of +/+ RNA gave rise to a band with four-fold as many counts as that from 20 μg of *shi/shi* RNA, indicating that the steady-state level of transcripts in the brains of 18-day-old shiverer mice is 16-fold lower than that in the brains of normal mice of the same age.

For analysis of the 3' end of exon I a 0.51 kb AvaII-PvuII fragment, 3'-labeled at the AvaII end (Figure 3A) was isolated and hybridized with test RNAs. The hybrids were digested with S1 nuclease and analyzed by autoradiography of

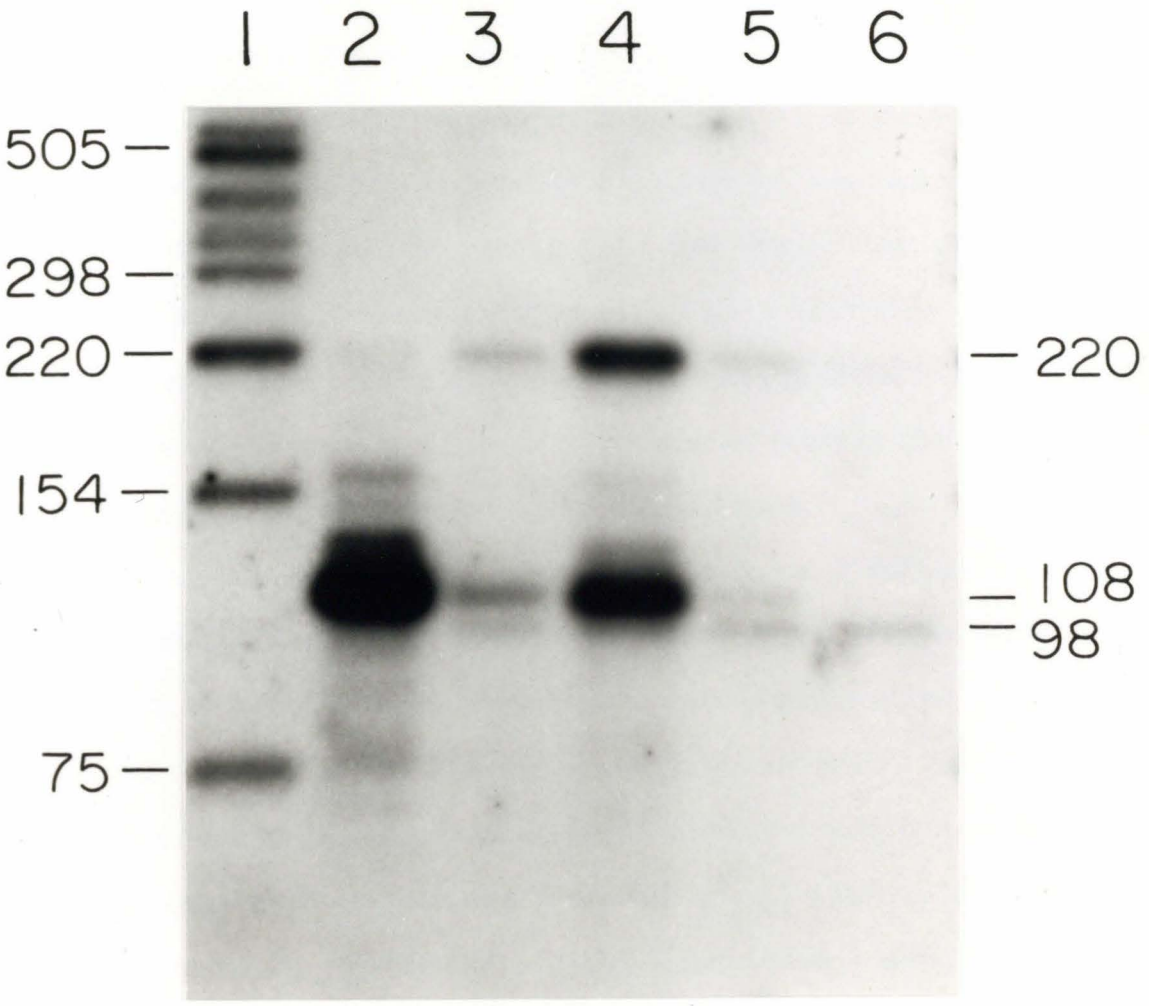
denaturing gels. Lane 5 of Figure 3B shows that +/+ RNA protected 110 bp from the labeled AvaII site; previous data have shown this to represent the 3' end of exon I, and results from protection by correctly spliced transcripts (see Chapter 2). While RNA from *shi/shi* brains protected the probe fragment in the 5' experiment, no protection is detectable with the 3' probe (lane 6), even at greater sensitivities than those required to detect the 5' protection. This indicates that the transcripts which are produced are not spliced at the correct site, i.e., the 3' end of exon I. Transcripts which are not spliced here may extend an indefinite distance 3' (i.e., rightward in Figure 3A) of the probe fragment. The possibility exists that an undetected fraction of the MBP transcripts in *shi/shi* RNA is spliced at the correct site, but careful comparison of different exposures of the gel presented in Figure 3B indicates that this fraction is less than one-fifth.

Protection of the full-length probe fragment in an S1 nuclease protection experiment can be the result of either the presence in the annealing mixture of RNA molecules which hybridize with the entire length of the probe fragment, or some DNA-DNA annealing which takes place after the dilution of the 80% formamide annealing mixture with aqueous S1 nuclease digestion buffer. The amount of protection due to DNA-DNA reannealing is variable from experiment to experiment. The protection of the full-length probe fragment in lane 6 of Figure 3B is consistent with the presence in shiverer brain RNA of a transcript which initiates somewhere 5' of the AvaII labeling site in the middle of exon I and continues into the first intron, past the PvuII site which defines the right end of the probe fragment. Since other data from this experiment indicate the presence in this RNA of transcripts which begin at the 5' end of the exon but which are not spliced at the 3' end of the exon, it is probable that some of the full-length fragments seen in lane 6 are protected by RNA molecules. The protection of the 220 bp probe fragment seen in lane 3 of Figure 3B may result from the presence in

shiverer brain of an RNA which initiates 5' of the PvuII site 5' of exon I, and continues through exon I. Such a transcript may also be present in the brains of normal mice, but is not detected when a four-fold lower amount of RNA is used in the annealing, as is the case in lane 2 of Figure 3B. It may also be due to DNA-DNA reannelaing which occurred to a greater extent in this incubation than in the others shown in this experiment (lanes 1 and 2). Further experiments would be required to determine whether such an RNA species is present in shiverer brains, and to discover its origin.

Since one aspect of RNA processing, i.e., splicing at the 3' end of exon I, has been shown to be defective in shiverer mice, and the normally used poly(A) addition signal at the end of exon VI (Chapter 2) is deleted in the shiverer genome, it is interesting to inquire whether the transcripts observed to initiate at the 5' end of exon I in shiverer mice are polyadenylated. Total brain RNA from 18-20 day shiverer mice was chromatographed on an oligo(dT) cellulose column, and the bound, poly(A) containing molecules eluted. 900 μg of total RNA gave 10 μg of poly(A) material. 1.0 μg of the poly(A) RNA and 1.0 μg of total RNA were used in an S1 protection experiment with the 0.22 kb PvuII-AvaII probe fragment described above. Both samples protected a similar, small amount of the probe fragment to a length of 108 nt (Figure 4, lanes 3 and 5). The weak band of length 98 nt appears in lanes 2-5 as well as lane 6 for which only carrier tRNA was used in the hybridization step. It does not appear in other experiments with these samples and it probably arises from a minor contaminant of the end-labeled probe fragment preparation used in this experiment, and is irrelevant to the question of MBP transcripts. 20 μg of total *shi/shi* RNA protects much more probe fragment (lane 4) than 1 μg of poly(A). This experiment indicates that MBP transcripts in shiverer mice are not greatly enriched in the poly(A) fraction, pointing to another defect in RNA processing caused by deletion of the 3' end of the MBP gene.

Figure 4. S1 protection experiment using poly(A) and total brain RNA from shiverer mice. Autoradiogram of 5% acrylamide, 50% urea gel in which products of the S1 protection experiment were electrophoresed. Lane 1, end-labeled DNA markers. Lanes 2-6, fragments remaining after protection of the 220 bp AvaII-PvuII fragment shown in Figure 3A with the following RNAs. Lane 2, 0.10 μg poly(A) RNA from brains of 18 day +/+ mice, plus 10 μg carrier yeast tRNA; lane 3, 1 μg poly(A) RNA from brains of 18 day *shi/shi* mice plus 9 μg carrier; lane 4, 20 μg total brain RNA From 18 day *shi/shi* mice; lane 5, 1 μg total brain RNA from 18 day *shi/shi* mice, plus 9 μg carrier RNA; lane 6, 10 μg carrier RNA only. Sizes are in nucleotides.


1
2
3
4
5
6
505
298
220
154
75
220
108
98

The implications of the transcription pattern described here are discussed below.

Discussion

The cloning of the MBP gene from wild type mice has made possible the characterization of the deletion previously reported to exist in the shiverer mouse. While the first exon and part of the first intron remain in mutant mice, all of the MBP gene 3' of the breakpoint, about 13 kb downstream from exon I, has been removed. Five exons, encoding 1.7 kb of the 1.9 kb of mRNA for 14 kd MBP, are lost with the deletion. The lengths of junction fragments observed indicate that there is new DNA present in the place of the deleted material, and that there has not been simply a loss of all DNA distal to the MBP gene.

Sequences flanking the first exon for 13 kb on the 3' side are not removed by the deletion. Analysis of transcripts present in the mutant indicates that qualitatively correct initiation occurs at the normal transcription start site, but that correct splicing at the 3' end of exon I does not occur. The downstream exons, to which splicing normally occurs, are missing, and it appears that no commonly used cryptic splice site (Treisman et al., 1983) is uncovered. As discussed previously, a rarely-used, 90 bp exon probably resides somewhere in intron I (Chapter 2). However, if it is still present in shiverer DNA, it does not participate in efficient splicing of MBP transcripts. MBP transcripts are rarely polyadenylated in the mutant, indicating that no efficiently used signal for cleavage and polyadenylation is uncovered or introduced by the deletion. The number of MBP transcripts initiating at the beginning of exon I is reduced by 16-fold in shiverer mice. It is not known whether this reflects less efficient initiation by the mutant gene, perhaps due to loss of a transcription-promoting element, or a reduced stability of the unprocessed or incorrectly processed RNAs.

These observations provide a basis for an understanding of many of the abnormalities displayed by the shiverer mouse. Since splicing and polyadenylation do not occur normally, MBP transcripts are expected to be large and heterogenous in size. They are also less abundant than in wild type mice, and would hybridize with only 180 bp out of 1462 bp of the pMBP-1 cDNA probe used in the RNA blots reported previously (Chapter 1). Therefore the study of transcription presented here would predict the failure to observe MBP mRNA in those experiments.

The absence of five out of six protein-coding exons explains the failure to observe MBP on gels of myelin proteins from shiverer brains (Ganser and Kirschner, 1980) or in vitro translations of brain RNA from shiverer mice (Campagnoni et al., 1984). There are several reports of the detection of low but significant amounts of MBP antigens in shiverer myelin by radioimmune assay (Dupouey et al., 1979; Ganser and Kirschner, 1980; Lachapelle et al., 1980). These reports are not inconsistent with our findings since the unspliced MBP transcripts we describe could, if they were to be translated, direct the synthesis of a peptide of 61 amino acids. The first 56 residues would be those encoded by exon I and may contain an antigenic site or sites sufficient to be detected by radioimmune assay, while the five residues at the C-terminus would be encoded by the first 15 nucleotides of intron I. The sixth triplet of intron I is a termination codon (see Chapter 2). Such a peptide would not be observed on gels because of its greatly reduced abundance, and its drastically altered mobility relative to authentic MBP.

It has been suggested that MBP plays a critical structural role in CNS myelin, possibly by an interaction between its basic and hydrophobic residues and the cytoplasmic face of the myelin membrane (Carnegie and Dunkley, 1975; Rumsby, 1978), and that its absence may account for the failure of myelin membranes to pack tightly in shiverer mice (Ganser and Kirschner, 1980). However, the reason for the lack of MBP was not known, and it could not be ruled

out that both the loss of MBP and other aspects of the phenotype might be due to some other, fundamental defect in myelination. By showing that the MBP gene maps to the same chromosome as the shiverer mutation, and by providing a detailed molecular model based on a characterization of the MBP deletion to account for some of the biochemical abnormalities in shiverer myelin, these data constitute further evidence to support the hypothesis that the MBP deletion is the primary lesion of the shiverer mutation. Since the 3' breakpoint of the deletion is not known, there may be other genes which are removed in part or in whole by this deletion. The shiverer mutation has been maintained for eleven years and has been crossed onto several inbred backgrounds, including B6C3H (Billings-Gagliardi et al., 1984) and C3H/SWV (Campagnoni et al., 1984). While the MBP deficiency and the other aspects of the shiverer phenotype have never been separated in these crosses, they would not be separable by homologous recombination if they were the result of the loss of two different genes in the same deletion event. No karyotypic studies of shiverer mice have been reported, and therefore we do not know whether or not the shiverer deletion is so large as to remove a visible portion of chromosome 18. The closest marker with respect to which shiverer has been mapped is syndactylism (*sy*). Twenty-four centimorgans separate *sy* from *shi* (Richard Sidman, Harvard University, personal communication), providing no indication of possible deletion size.

A number of inductive influences may regulate aspects of oligodendrocyte development, including cell proliferation, recognition of axons, induction of myelin proteins, and size of sheath assembled (for review, see Bray et al., 1981). There may be oligodendrocyte-expressed genes which play roles in these interactions, and it is conceivable that one such gene is inactivated or lost in the deletion, preventing compaction of central myelin. No specific candidates for such a gene product can be proposed, however, and it does not appear to be

necessary to propose the deletion of another gene or genes to account for the shiverer phenotype. The near total absence of the major dense line in shiverer CNS myelin is not surprising since MBP has been shown to be localized to this structure in normal myelin. The few regions of major dense line observed in shiverer CNS myelin may be due to the action of the basic cytoplasmic tail proposed for proteolipid protein, which represents 50% of CNS myelin protein and which may be capable of interactions similar to MBP (Laursen et al., 1984). Predictions regarding the effects of a lack of MBP on the ultrastructure and accumulation of myelin cannot be made rigorously since events in myelinogenesis are incompletely understood, and the role of MBP is not certain. It is reasonable to suggest that the complete loss of one major component severely disrupts the formation of myelin and thereby leads to the greatly reduced amounts of myelin and abnormal structure of the remaining sheaths in shiverer mice.

Despite the disastrous effects of the absence of MBP on central myelin, PNS myelin shows only minor morphological abnormalities in the shiverer mouse (Rosenbluth, 1980b). Recent unpublished experiments by Greg Lemke and Richard Axel of Columbia University suggest a reason for this. PNS has less MBP than CNS (Greenfield et al., 1973), and the transmembrane glycoprotein P_0 represents over 50% of total PNS myelin protein (Greenfield et al., 1973). DNA sequence analysis of a cDNA clone representing P_0 from mouse indicates that P_0 protein has a long, highly basic cytoplasmic tail that may serve the role that MBP in the CNS probably does, of maintaining the tightly wrapped structure of mature myelin by an interaction with the cytoplasmic faces of the wrapped membrane (personal communication). This model would explain the relative independence of MBP that PNS myelin morphology shows, and offers an element to be tested and included in or rejected from our understanding of myelinogenesis.

These data show that it is very possible that the MBP deletion is the primary lesion of the shiverer mutation. The establishment of a closer linkage between the shiverer locus and the MBP gene would be supportive of this hypothesis, and should be attempted. A further characterization of the MBP deletion to determine whether any other, closely-linked genes are missing would also be an important test of the hypothesis.

Experimental Procedures

Genomic Blotting

DNA was prepared from mouse livers by the method of Blin and Stafford (1976). Gels were run and partially depurinated essentially as described by Wahl et al. (1979) followed by blotting as described by Southern (1975). Hybridization probes were prepared by nick-translation (Rigby et al., 1977) of gel-purified restriction fragments derived by digestion of subclones of regions of the mouse MBP gene with the restriction endonucleases indicated. Hybridization conditions were as described by Wahl et al. (1979). The restriction fragment used for probe 1 was obtained by HindIII digestion of pH-F, a subclone of the 3.6 kb HindIII fragment containing exon I of the MBP gene, in pUC8. Probe 2 was obtained by EcoRI digestion of a subclone of the 1.4 kb EcoRI fragment from the first intron, in pBR325. Probe 3 was obtained by EcoRI digestion of pE-2/4, a subclone of the 16 kb and 3.5 kb EcoRI fragments from the middle of the gene, in pBR325. Probe 4 was isolated from a SalI + EcoRI digest of pE-2/4. The 3.8 kb BamHI-SalI fragment used as probe in the chromosomal mapping experiments was isolated from a BamHI + SalI digest of pE-2/4.

RNA Preparation

RNA was prepared from brains as described by Chirgwin et al. (1979). Concentration was determined by UV spectrophotometry, and intactness by electrophoresis through agarose-methylmercury hydroxide gels (Bailey and Davidson, 1976) and staining with ethidium bromide.

S1 Nuclease Protection Experiments

The 0.22 kb AvaII-PvuII fragment used in analysis of the 5' end of MBP transcripts was obtained by digesting subclone pH-F with AvaII, 5' end-labeling by the method of Maxam and Gilbert (1980), followed by elution of the 0.28 kb AvaII fragment from a 5% polyacrylamide gel, digestion with PvuII, and isolation of the 0.22 kb AvaII-PvuII fragment from a second gel.

The 0.52 kb AvaII-PvuII fragment used in analysis of the 3' end of exon I was obtained in a similar manner, differing in that 3' end-labeling was used, and the 0.58 kb AvaII fragment extending from the middle of exon I into intron I was eluted from the first gel, and the 0.52 kb band from the second.

Annealing conditions in 80% formamide were as described by Favaloro et al. (1980). The strand separation temperature for each end-labeled probe fragment was determined empirically, and DNA-RNA annealing was allowed to occur at 1-3°C higher. Optimal S1 nuclease concentrations were also determined experimentally. The products of S1 nuclease digestion were electrophoresed through 50% urea, 5% acrylamide gels after denaturation in 10 mN NaOH, 90% formamide, 90°C.

Radioactivity in gel slices was determined by solubilizing slices of equal size in NCS tissue solubilizer (Amersham), followed by scintillation counting with Hydrofluor scintillation cocktail (National Diagnostics). The value obtained by counting the 108 nt region from lane 1 of the gel in Figure 3B was subtracted from the values for lanes 2 and 3. Counts per minute for each sample were: lane 1, 35.5±5%; lane 2, 106.9±2%; lane 3, 53.0±3%.

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