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The Dysmyelinating Mouse Mutations Shiverer (*shi*) and Myelin Deficient (*shi^{mld}*)

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Shiverer (shi/shi) is an autosomal recessive mouse mutation that produces a shivering phenotype in affected mice. A shivering gait can be seen from a few weeks after birth until their early death, which occurs between 50 and 100 days. The central nervous system of the mutant mouse is hypomyelinated but the peripheral nervous system appears normal. The myelin of the CNS, wherever present, is not well compacted and lacks the major dense line. Myelin basic protein (MBP), which is associated with the major dense line, is absent, and this is due to a deletion of the major part of the gene encoding MBP. Transgenic shiverer mice that have integrated and express the wild-type mouse MBP transgene no longer shiver and have normal life spans. Conversely, normal mice that have integrated an antisense MBP transgene, shiver. Myelin deficient shi^{mld}/shi^{mld} is allelic to shiverer (shi/shi) but the mutant mouse is less severely affected. Although MBP is present in the CNS, it is low in quantity and is not developmentally regulated. The gene encoding MBP has been both duplicated and inverted. Transgenic shi^{mld}/shi^{mld} mice with the wild-type MBP transgene have normal phenotypes.

KEY WORDS: shiverer; myelin deficient; myelin basic protein; hypomyelination; myelin.

INTRODUCTION

Shiverer (*shi/shi*) and myelin deficient (shi^{mld}/shi^{mld}) are mutations of the myelin basic protein structural gene (MBP). These mutations result in the absence of MBP in the case of the shiverer mutant and very low levels in the myelin deficient mutant. MBP is a major protein component of central nervous system (CNS) myelin; and diminished levels of it result in poorly compacted and sparse

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myelin. In both the shiverer and the myelin deficient mutant mouse, the CNS is hypomyelinated but the peripheral nervous system (PNS) is normal. MBP is less abundant in the PNS and apparently plays a less prominent role.

These mutants are phenotypically very similar, both exhibiting a trembling gait, convulsions, and an early death, between 50 and 100 days. Time of death is somewhat dependent on the genetic background of the mutant.

PHENOTYPE

Shiverer

The first shiverer mutant mouse was observed by Biddle *et al.* (1973) in the F38 generation of Swiss Vancouver stock. Shiverer (*shi/shi*) is an autosomal recessive trait in the mouse and is characterized by generalized tremors, seizures, and a shortened life span (Chernoff, 1981). Affected animals are indistinguishable from normal littermates at birth, but at about 12 days of age mutant mice develop a generalized tremor, resembling a shiver, that accompanies movement by the animal. When the animals are at rest, no tremors can be seen. From about 30 days onward, the mice have tonic seizures that increase in frequency and severity. Attacks begin with the animal tucking its head and forelimbs into the chest, arching its back, and extending the hind limbs. At the height of the attack, breathing becomes rapid and the animal enters a rigid ventral arch. After several seconds in this position, the limbs relax and the animal gets up and walks about in a dazed condition for several minutes. Several types of stimulation including sound, motion, light, and handling can initiate attacks. Stimulation by shaking the cage at 5-min intervals increases the frequency from a base rate of 1.5 attacks per h to 4.4 per h. The mean duration of these attacks is 20 s, and treatment with lithium, vitamin B6, diphenylhydration, or ethanol does not affect the rate or duration of clinical symptoms (Chernoff, 1981).

Myelin Deficient

The *shi*^{mld}/shi^{mld} mutant mouse first was observed by Doolittle and Schweikart (1977) in a strain of MDB/Dt mice at Purdue. It is an autosomal recessive mutation characterized by a deficiency of central nervous system myelin. Shiverer and myelin deficient were shown to be alleles because they failed to complement each other in heteroallelic animals (Cowan, 1980; Doolittle *et al.*, 1981; La Chapelle *et al.*, 1981; Akowitz *et al.*, 1987) and because both mutations are located at the distal end of chromosome 18 (Sidman *et al.*, 1985). Molecular studies have shown that the gene encoding MBP has been mutated in *shi*^{mld}/

shi^{mld} mice (Akowitz *et al.*, 1987; Okano *et al.*, 1988a; Popko *et al.*, 1987, 1988).

The shivering motion characteristic of these animals was not apparent until 12 days of age. From 12 to 15 days until their death, myelin deficient mutant mice exhibit a rapid trembling motion of the limbs and the caudal trunk during locomotion. Convulsions begin to occur between 30 and 60 days and can be triggered by noise or slight mechanical shocks such as jogging the cage. Mice die between 30 and 60 days (Matthieu *et al.*, 1984). The life expectancy of the mutant mice studied by Matthieu differ from *shi*^{mld}/*shi*^{mld} animals on the B6C3 (C57BL/6J × C_3H/HeJ F_1) stock (Shen *et al.*, 1985). Animals with the *shi*^{mld}/*shi*^{mld} mutation on the B6C3 hybrid background have tremors that are recognizable between 16 and 18 days. In the second month of life, affected animals have convulsions that persist throughout life. They die between 150 and 200 days, although occasionally animals survive longer (e.g., 288 days) (Shen *et al.*, 1985).

MORPHOLOGY OF MYELIN

Myelin is a multilamellar sheath laid down along selected nerve fibers that facilitate saltatory conduction of stimuli from the nerve-cell body to its target (Huxley and Stampfli, 1949). When myelin is present, action potentials are generated at successive nodes of Ranvier and the myelin-covered internodes are silent because of the insulating qualities of myelin (review by Ritchie, 1984). The axolemma is also different in myelinated axons, and this is thought to be due to specialized contacts with glial cells (Rosenbluth, 1988). While myelin is present in some invertebrates such as annelids and crustaceans, it is most commonly associated with the vertebrate nervous system (Waehneldt *et al.*, 1986). In mammals, myelin of the central nervous system is elaborated by oligodendrocytes and in the peripheral nervous system by Schwann cells. Flattened processes from these accessory cells wrap in a spiral fashion around a segment of an axon, and eventually the multilamellar structure becomes compacted, losing much of the cytoplasmic content. The cytoplasmic faces of the oligodendrocyte membrane become closely apposed to form the major dense line (MDL), while the outerfaces of the cell membrane lie together, forming the less optically dense interperiod line. The myelin sheath thus forms a multilamellar structure with a major dense line alternating with the interperiod line. The periodicity of the lamellae is 11.5–12 nm in peripheral myelin and 10.5–11.5 nm in central axons (Raine, 1984). Described in molecular terms, myelin is a lipid bimolecular lipid leaflet sandwiched between two layers of protein.

Although myelin of the central and peripheral nervous system is morpho-

logically and biochemically distinct, electrophysiological characteristics of the myelinated CNS and PNS fibers are essentially the same.

Myelin in the Shiverer Mutant Mouse

In the dysmyelinating mouse mutant, shiverer, light microscopy revealed an almost total lack of myelin in brains of affected mice of all ages; when compared to littermate controls (Bird *et al.*, 1978). There is no evidence of gliosis, sudanophilia, and macrophage infiltration (Bird *et al.*, 1978), and therefore, dysmyelination does not result from the destruction of oligodendroglia. Electron microscopic studies of the shiverer CNS reveal that many axons, including large-caliber axons, are unmyelinated, and myelin, where it does occur, is reduced in amount and variable in compactness (Privat *et al.*, 1979; Kirschner and Ganser, 1980; Rosenbluth, 1980). Normal mouse oligodendrocytes form broad sheet-like processes that surround the axon, but in the *shi/shi* mutant numerous fine tubular processes are formed (Rosenbluth, 1980a). Axoglial junctions in the shiverer CNS are present in aberrant locations, and they are bizarre in orientation and form, and most surprisingly, they are increased in number despite the overall reduction in myelin (Rosenbluth, 1981). The most characteristic feature of the abnormal CNS myelin is the absence of the major dense line formed by the apposition of cytoplasmic faces of the oligodendrocyte membrane (Privat *et al.*, 1979; Kirschner and Ganser, 1980). Correlated with this observation are biochemical studies which showed that myelin basic protein (MBP) is also absent in the shiverer central nervous system (Dupouey *et al.*, 1979; Kirschner and Ganser, 1980). Therefore, it was concluded that MBP is localized in the cytoplasmic leaflet of the oligodendrocyte membrane and is essential for formation of the major dense line (Dupouey *et al.*, 1979; Privat *et al.*, 1979; Ganser and Kirschner, 1980; Mikoshiba *et al.*, 1981; Barbarese *et al.*, 1983).

Shiverer peripheral myelin is normal in thickness, lamellar structure, and periodicity (Kirschner and Ganser, 1980; Rosenbluth, 1980b). Subtle abnormalities do occur, however, such as cytoplasm between lamellae which contains fine filamentous matrix not normally seen. The terminal loop of the innermost lamella of the sheath, which is often larger than normal and filled with the same fine filamentous material, may indent the axon conspicuously (Rosenbluth, 1980b). This suggests that MBP plays other roles in myelination besides that of compacting the lamellae.

Myelin in the Myelin Deficient Mutant Mouse

Electron microscopic studies of the optic nerve from 15-day-old shi^{mld}/shi^{mld} mice showed that myelin formation clearly was reduced compared to that of

age-matched controls. Some of the oligodendrocyte processes showed large amounts of cytoplasm, and no major dense line was observed (Matthieu *et al.*, 1984). In older (84-day-old) mutant animals, the thickness of the myelin sheaths had increased to 10–20% of normal, and some of the myelin had formed a major dense line (Matthieu *et al.*, 1984).

In the PNS, the ratio between thickness of the myelin sheath and caliber of the axon did not differ from that in control mice and no difference in structure and periodicity of the myelin lamellae was observed (Matthieu *et al.*, 1984).

MYELIN PROTEINS

A method for purifying myelin made detailed biochemical studies possible (Norton and Podulso, 1973). The protein composition of myelin is relatively simple, and the major proteins are myelin-specific in both the CNS and the PNS.

In the CNS, proteolipid protein (PLP) and myelin basic protein (MBP) account for 60–80% of the protein, but a heterogeneous population also can be identified by SDS-PAGE analysis (Lees and Brostoff, 1984). Proteolipid protein PLP (MW = 30 kd) represents 50% of the total protein in CNS myelin. The gene encoding PLP is located on the X chromosome (Willard and Riordan, 1985; Dautigny *et al.*, 1985) and is made up of seven exons spanning 15 kb (Diehl *et al.*, 1986). Distribution of hydrophobic and hydrophilic segments deduced from the amino acid sequence suggests a complex trans-membrane disposition, in which PLP spans the membrane several times (Stoffel *et al.*, 1984; Lees and Brostoff, 1984; Milner *et al.*, 1985). A second myelin proteolipid, DM20, is translated from an alternatively spliced PLP message (Nave *et al.*, 1987). The N-terminal methionine of PLP is removed posttranslationally, and residue 198 and other residues are acetylated (Stoffyn and Folch-Pi, 1971).

Myelin basic protein comprises 30–40% of the protein content of CNS, and 5–15% of PNS, myelin. Rats and mice have four forms of MBP with similar amino acid sequences but different molecular weights, 21.5, 18.5, 17.0, and 14.0 kd, and these forms are represented at the ratio of 1:10:3.5:35, respectively (Martenson *et al.*, 1972; Barbarese *et al.*, 1977, 1978). The 18.5-kd form of human (Carnegie, 1971) and bovine (Eylar *et al.*, 1971) MBP was sequenced and found to be essentially the same as the 14-kd form of rat MBP. The small, 14-kd protein differs from the 18.5-kd form in that 40 amino acid residues are absent from near the carboxy-terminal end of the molecule (Dunkley and Carnegie, 1974). Tryptic peptide maps of the four basic proteins show that the 21.5- and the 17-kd species contain peptides not found in the 18.5- and 14-kd species (Barbarese *et al.*, 1977). Thus, the 21.5-kd species contain all the amino acid sequences found in other basic proteins, and the lower molecular weight proteins have either sequences deleted at a position close to the N-terminus (18.5-kd

MBP) or 40 residues near the carboxy-terminal end (17-kd MBP) or both (14-kd MBP).

A group of myelin proteins, known as Wolfgram proteins (Wolfgram, 1966), is more prominent in CNS, than PNS, myelin. Some components of the Wolfgram proteins are enzymes such as 2′,3′-cyclic nucleotide 3′-phosphodiesterases (EC 3.1.4.37; CNPs). CNPase activity has been attributed to two proteins, CNPI and CNPII, which together represent about 5% of CNS myelin proteins and somewhat less in the PNS (Drummond and Dean, 1980; Sprinkle *et al.*, 1980). Myelin-associated glycoprotein (MAG) is present in low quantities (1%) (Quarles, 1980) and is thought to be important for the process of contact and adhesion between nerve axon and myelinating oligodendrocyte (for review see Sims, 1988).

In the PNS, P_o(MW 31.5 kd) is the most abundant protein (>50% of total myelin protein) and is unique to Schwann cells (Singh and Spritz, 1974; Wiggins *et al.*, 1975; Ishaque *et al.*, 1980). The sequence of P_o (Lemke and Axel, 1985) shows that it is a member of the immunoglobulin superfamily of proteins (for review see Lai *et al.*, 1989). Homotypic interactions characteristic of this family probably play a role in compaction of both internal and external surfaces of PNS myelin (Lemke and Axel, 1985; Saavedra *et al.*, 1989). Other proteins, such as MBP and CNPs, are present in PNS myelin but at lower concentrations than in CNS myelin. MAGs are present in both CNS and PNS myelin. PLP is found in Schwann cells and their tumors, as shown by immunofluorescence microscopy and Western blot analysis (Puckett *et al.*, 1987). Although PLP message can be detected in rat and rabbit sciatic nerve, the translated product is not incorporated into the myelin membrane (Puckett *et al.*, 1987).

Since myelin is a periodic structure, the myelin proteins probably are integrated into membranes in precise pattern. Although disposition of myelin proteins is not fully understood, their absence results in a poorly constructed myelin sheath. Myelinating cells must orchestrate insertion of these polypeptides into the membrane. Synthesis of myelin components, their assembly into myelin sheath, has been reviewed extensively (Benjamin and Smith, 1984). *In vivo* labeling experiments have shown that newly synthesized MBP and CNPs appear in the myelin fraction without any lag, but PLP does not appear until 30 min later (Benjamin *et al.*, 1978; Colman *et al.*, 1982). It is suggested that MBP and CNP are discharged from free polysomes and incorporated into the myelin sheath, while the mechanism by which PLP is transferred to the membrane involves synthesis on membrane-bound ribosomes followed by a relatively slow transfer through an intracellular pathway (Colman *et al.*, 1982). The intracellular distribution of these myelin protein mRNAs in oligodendrocytes is very different, as has been shown by *in situ* hybridization studies (Trapp *et al.*, 1987, 1988a).

Myelin Proteins in the Shiverer and Myelin Deficient Mutant Mouse

MBP was measured in total brain homogenates by radioimmunoassay and shown to be absent in shiverer mutant mice (Depouey *et al.*, 1979; Ganser and Kirschner, 1980; Jacque *et al.*, 1983). Zonal centrifugation of shiverer brain homogenates showed no apparent myelin peak within the density that normally includes myelin; a peak was present at approximately 0.85–0.90 *M* sucrose, but it was devoid of MBP (Bourre *et al.*, 1980).

In shi^{mld}/shi^{mld} mutant mice 25 days of age, levels of MBP were 2% of normal, while at 90 days they reached 5% of control values (Jacque *et al.*, 1983; Matthieu *et al.*, 1984). The percentage of total brain CNP in purified myelin varied only slightly from controls, and PLP was decreased at early developmental stages in shi^{mld}/shi^{mld} myelin (60% of control values at 30 days) but increased to normal values later in life (93% of control values at 135 days) (Matthieu *et al.*, 1984).

GENES ENCODING THE MYELIN PROTEINS

The genes encoding myelin proteins, such as MBP (Takahashi *et al.*, 1985), PLP (Diehl *et al.*, 1987), MAG (Lai *et al.*, 1987), and P_0 (Lemke *et al.*, 1988), have been sequenced or mapped. cDNAs representing the five forms of MBP (Roach *et al.*, 1983; deFerra *et al.*, 1985; Newman *et al.*, 1987), PLP (Milner *et al.*, 1985), P_0 (Lemke and Axel, 1985), MAG (Milner and Sutcliffe, 1983; Arquint *et al.*, 1987; Salzer *et al.*, 1987), and CNP (Bernier *et al.*, 1987) have been sequenced. Extensive reviews have been written on the structural genes of myelin proteins (Lemke, 1987; Compagnoni, 1988; Sutcliffe, 1988a) and the mRNAs transcribed from them (Sutcliffe, 1988b). Since the gene encoding MBP is relevant to the *shi/shi* and shi^{mld}/shi^{mld} mutations, it is described here in detail.

The Gene Encoding MBP

The gene encoding mouse MBP was isolated from a cosmid library and mapped, and the exons were sequenced (Roach *et al.*, 1983; Takahashi *et al.*, 1985) (Fig. 1). It is composed of seven exons varying in size from 92 to 1500 base pairs. The exons are spread over a total distance of 32 kb, and the intron between exon II and exon III, being the largest, is 15 kb (Fig. 1). By comparing protein sequence data with DNA sequences, it was possible to postulate that the four different forms of MBP are generated by alternative splicing of exons (Takahashi *et al.*, 1985). The 21.5-kd form of MBP is encoded by all seven exons, while the 18.5-kd form is encoded by all exons except exon II, the 17-kd form of MBP is encoded by all exons except exon VI, and the low molecular

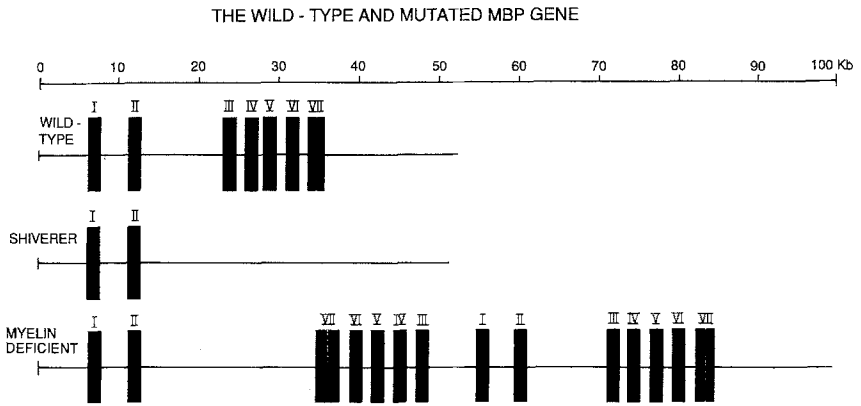

Fig. 1. Myelin basic protein gene (MBP) in wild-type, shiverer *(shi/shi)*, and myelin deficient *(shi^{mld}/shi^{mld})* mutant mice. Exon sites are exaggerated and are not proportional. The normal MBP gene has seven exons which are spread over a 32-kb distance (Takahashi *et al.*, 1985). The shiverer MBP gene has a deletion (Roach *et al.*, 1983, 1985), and the myelin deficient mutant has a duplication and an inversion (Okano *et al.*, 1988; Popko *et al.*, 1988).

weight MBP (14 kd) is encoded by exons I, III, IV, V, and VII. cDNA clones encoding the four different forms of mouse MBP have been isolated and sequenced (deFerra *et al.*, 1985). Another cDNA that encodes a fifth, 17.2-kd form of MBP has been isolated (Newman *et al.*, 1987). This message has been generated by splicing of all the exons except exons II and V and appears to be less abundant than the message encoding the 17.0-kd form of MBP. Since the 17.2-kd form of MBP comigrates with the 17.0-kd form on a $NaDodSO_4$/polyacrylamide gel, it remained undetected until now. The mechanism for differential splicing is unknown, as is the function of the four forms of MBP.

The Mutated *shi/shi* MBP Gene

Shiverer mutant mice have no detectable MBP in the central nervous system (Dupouey *et al.*, 1979; Kirschner and Ganser, 1980). Similarly no message corresponding to MBP was found in brains of shiverer mice (Roach *et al.*, 1983). In shiverers, the structural gene encoding MBP contains a deletion with only the first two exons remaining (Roach *et al.*, 1985) (Fig. 1). The deletion extends from a region between the second and the third exon to 2 kb 3′ of the last exon (Molineaux *et al.*, 1986); the break point has been sequenced (Molineaux *et al.*, 1986).

The Mutated *shi^{mld}/shi^{mld}* MBP Gene

Shi/shi and *shi^{mld}shi^{mld}* are alleles, as has been shown by the genetic studies (Cowan, 1980; Doolittle *et al.*, 1981; La Chapelle *et al.*, 1981; Akowitz *et al.*, 1987). Low levels of MBP message were found in brains of myelin deficient mice, but these are not developmentally controlled (Matthieu *et al.*, 1984; Roch *et al.*, 1986; Popko *et al.*, 1987). The MBP gene in *shi^{mld}/shi^{mld}* mice has undergone a duplication and an inversion (Akowitz *et al.*, 1987; Okano *et al.*, 1988a; Popko *et al.*, 1987). Mapping of cosmid clones that extend over the area of the duplication reveals a tandem duplication of the MBP gene (Popko *et al.*, 1988; Okano *et al.*, 1988a). The 5′ MBP gene has an inversion so that the order of the exons is I, II, VII, VI, V, IV, and III, and the downstream MBP gene, which is 7 kb 3′ to the inverted gene, appears from mapping studies to be normal (Popko *et al.*, 1988) (Fig. 1).

DEVELOPMENTAL CONTROL OF MBP GENE EXPRESSION

Oligodendrocytes form myelin sheaths in the CNS. These cells first can be distinguished at birth and arise from the progenitor cell O-2A (Raff *et al.*, 1983). O-2A cells differentiate *in vitro* in response to the trophic factors PDGF (platelet-derived growth factor) (Raff *et al.*, 1988) and CNTF (ciliary neurotrophic factor) (Hughes *et al.*, 1988). Myelination in the CNS takes place neonatally, progressing in a caudal to rostral fashion. Myelination begins at about 7 days postnatally and reaches a high level at around 18 days; after this time myelination levels off (for a review see Gould, 1985). Messenger RNA corresponding to MBP follows a similar time course (Jacque *et al.*, 1983; Carson *et al.*, 1983; Zeller *et al.*, 1984). MBP message first can be detected by Northern blot analysis at around 7 days in the neonate, and it reaches the highest levels at around 18 days. By 90 days, it has dropped to steady-state levels (Zeller *et al.*, 1984; Popko *et al.*, 1987). Expression of MBP mRNA can be detected as early as 6 h after birth in the medulla oblongata using *in situ* hybridization techniques (Verity and Campagnoni, 1988).

Developmental Expression of MBP in Mutant Mice

In the shiverer mutant mouse, MBP mRNA was not detectable (Roach *et al.*, 1983), presumably because the transcript from the deleted gene is unstable. In the myelin deficient mutant mouse, the MBP gene is not correctly developmentally expressed. Low levels of message are detected at 7 days but levels do not peak at 18 days; instead they increase steadily until 3 months of age, when they reach a maximum that is 10% that of a normal age-matched adult (Roch *et al.*, 1986; Popko *et al.*, 1987).

Several explanations for this pattern of expression are possible. The first is that transcripts from the inverted MBP gene form antisense RNA, which then interacts with message from the normal MBP gene (Okano *et al.*, 1988b; Popko *et al.*, 1988). Antisense MBP mRNA, in fact, has been detected (Okano *et al.*, 1988b; Popko *et al.*, 1988).

Another possibility is that transcription of the normal downstream MBP gene is prevented, because the upstream MBP gene has lost its termination codon (it is inverted) and forms a long transcript that interferes with initiations of transcription. Most likely, both these mechanisms contribute to the low levels of MBP in the myelin-deficient mouse.

LIPID COMPOSITION OF CNS MYELIN

An extensive survey of the lipid composition of myelin in mutant mice with myelin deficiencies (*jp/Y, jp^{msd}/Y, qk/qk, shi/shi,* and *shi^{mld}/shi^{mld}*) shows that lipids in the CNS, expressed as a percentage of control levels, are strikingly similar in all five mutants (Ganser *et al.*, 1988a,b). Glycolipids, which are the lipid classes most concentrated in myelin, show reductions of greater than 70–85% in *shi/shi* and *shi^{mld}/shi^{mld}*. Phospholipids are reduced by 15–50% in these mutants, and cholesterol is diminished to 60% of control levels. In the PNS of *shi/shi* and *shi^{mld}/shi^{mld}*, the most prominent changes are an increase in sulfatide and loss of sphingomyelin (Ganser *et al.*, 1988a). Since the primary defect in shiverer and myelin-deficient mutants is the structural MBP gene, changes in lipid composition of the myelin sheath is a secondary effect. These data demonstrate that the production of myelin is a highly synchronized process and that deficiencies in one element lead to deficiencies in others.

TRANSGENIC ANIMALS

Recent biotechnology has made it possible to transfer genes into the mammalian genome (Gordon *et al.*, 1980; Constantini and Lacy, 1981). Gene regulation now may be studied in the context of the whole animal (for a review see Jaenish, 1988). This is a particularly unique technique for study of the nervous system, where the molecular basis of development and function is complex (for reviews see Rosenfeld *et al.*, 1988; Popko *et al.*, 1989). Genetically engineered animals also have a number of practical uses, such as models for human diseases or producers of therapeutic agents or improved meat and dairy products (for a review see Westphal, 1989).

The technique for generating transgenic animals has been described in an excellent manual (Hogan *et al.*, 1986), and many of the important parameters have been explored and discussed (Brinster *et al.*, 1985, 1988).

Eggs are harvested from the mouse oviduct about 16 h after fertilization, when

the male and female pronucleus have swollen but not yet fused. DNA (about 200 copies) is injected into one of the pronuclei. The eggs are allowed to develop to the two-cell stage and then are transferred to a pseudopregnant foster mother. Pups born to the foster mother are screened for the presence of the transgene. At the time of weaning, a small piece of tail is excised from each pup and DNA is extracted. The DNA is screened for the presence of the transgene by Southern blot analysis or polymerase chain reaction (PCR) (Abbott *et al.*, 1988). Ten to thirty percent of pups born have integrated injected DNA into the genome.

Correction of Shiverer Mutant Mouse Phenotype

We introduced the wild-type MBP gene into the genome of shiverer mice in order to test the hypothesis that abnormal myelination, shivering, seizures, and shortened life span all were due to a simple deletion in the shiverer MBP gene. Shiverer mice homozygous for the MBP transgene were phenotypically and behaviorally normal; the central nervous system was myelinated, and the myelin had a major dense line (Readhead *et al.*, 1987).

A circular cosmid clone containing the wild-type mouse MBP gene, as well as 4 kb of 5′ and 3 kb of 3′ flanking sequence (Takahashi *et al.*, 1985), was microinjected into the pronucleus of fertilized eggs. The MBP transgene was expressed normally during development; MBP mRNA first was detected 7 days after birth, reached high levels at 18 days, and then fell to steady-state levels by 90 days. This pattern was identical to the one seen in normal mice. SDS-PAGE analysis showed that all four molecular weight forms of MBP were present at the correct ratio, indicating that mechanisms for splicing were present in shiverer transgenic mice. One noticeable difference was the low level of expression of the MBP transgene compared to the normal gene. Shiverer mice homozygous for the MBP transgene (*shi/shi*; MBP′/MBP′) had levels of MBP that were about 25% of normal. This was both at the RNA level, as judged by hybridizing intensity on Northern blots, and at the protein level, as measured by radioimmunoassay. The low level of expression could be due to the site of integration (Brinster *et al.*, 1985) or to the absence of enhancer regions situated more than 4 kb 5′ or 2 kb 3′ of the MBP gene. Some enhancer sequences have been found more than 40 kb from the gene (Grosveld *et al.*, 1987). In spite of the low level of MBP in transgenic shiverer mice, the normal phenotype was restored and compact myelin with a major dense line was detected in the CNS (Fig. 2).

Correction of the Myelin Deficient Mutant Mouse Phenotype

The MBP transgene was crossed into myelin deficient mutant mice by mating them with homozygous transgenic shiverer mice. Myelin deficient mice

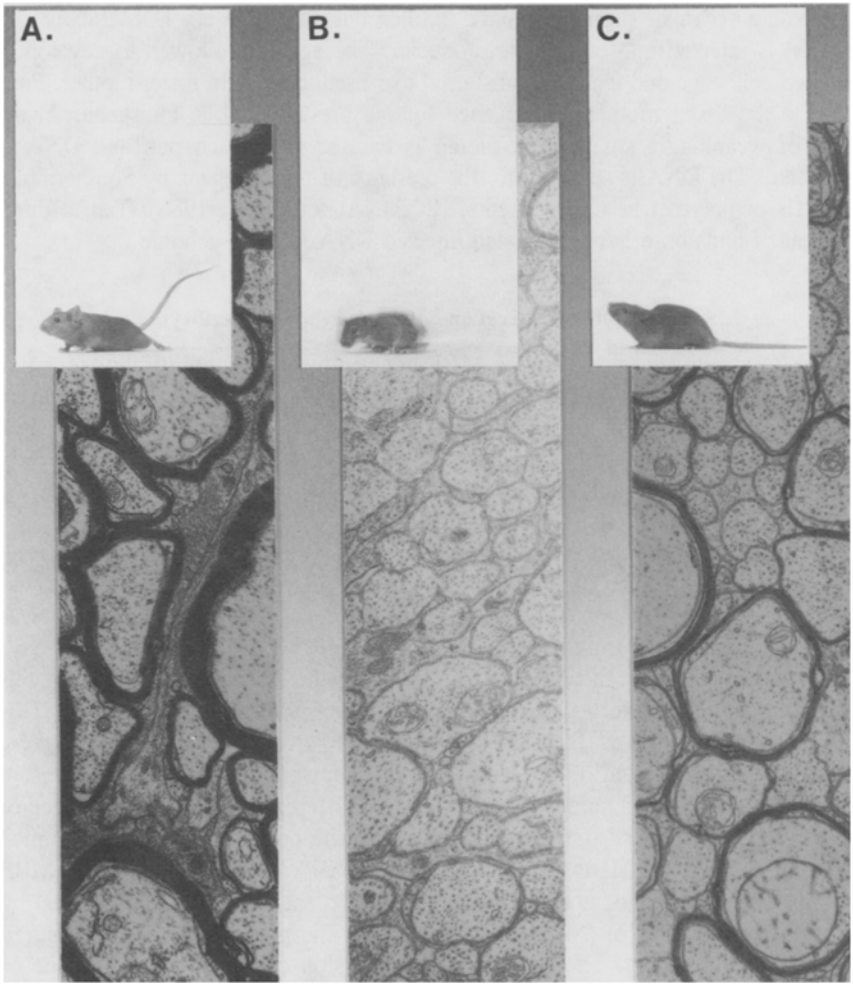

Fig. 2. Electron micrographs of cross sections from the Prechiasmatic optic nerve in wild-type, shiverer, and transgenic shiverer mice. (A) A wild-type mouse, in which multilayered myelin is wrapped around all axons. (B) A shiverer mouse (*shi/shi*), in which compact myelin around axons is completely absent. A few axons are enveloped in loose oligodendrocytic lamellae. (C) A transgenic shiverer mouse that is homozygous for the normal MBP transgene. A greater number of axons are myelinated than in shiverer mice, but the myelin sheath is not as thick as in wild-type mice.

homozygous for the MBP transgene had a normal phenotype (Popko *et al.*, 1987). This work has answered a number of questions. First, it confirmed genetic studies that had shown that myelin deficient and shiverer were alleles

(Cowan *et al.*, 1980; LaChapelle *et al.*, 1981; Akowitz *et al.*, 1987); and second, it showed that low levels of MBP in the myelin deficient mutant mouse are not due to defects in transcriptional or translational machinery but that the defect probably lies in the nature of the mutation itself. The inverted MBP gene produces antisense mRNA in myelin deficient mice (Popko *et al.*, 1988), but the antisense RNA has little, if any, effect on the level of expression by the MBP transgene. This could be because it is combining with messages from the endogenous normal MBP gene.

By different crosses, we are able to produce mice with graded levels of MBP, ranging from 0% in shiverer to 125% in normal mice that are homozygous for the transgene (Popko *et al.*, 1987) (Table I). These mice will be useful in physiological and pharmacological studies.

Conversion of a Normal to a Shiverer Phenotype

Transgenic mice with an anti-sense MBP transgene displayed a shivering phenotype (Katsuki *et al.*, 1988). A minigene was constructed, which consisted of the mouse MBP promoter region approximately 1.3 kb in length, a portion of the rabbit B-globin gene, the mouse MBP cDNA in the antisense orientation, and the polyadenylation sites of rabbit β-globin and simian virus 40 early genes (Katsuki *et al.*, 1988). Transgenic *shi*/+ mice with the antisense MBP transgene

Table I. Transgenic Mutant Mice and Their Phenotypes

Genotype	Percentage of normal MBP mRNA[a]	Phenotype	Life span (months)
shi	0	Shivering, convulsions	2–5
shi/mld	1	Shivering, convulsions	N.D.[b]
mld/mld	2	Shivering, convulsions	2–5
shi/shi; MBP'/–	12.5	Shivering, convulsions	6
shi/mld; MBP'/–	13.5	Shivering, convulsions	N.D.
mld/mld; MBP'/–	14.5[c]	Shivering[d]	N.D.
shi/shi; MBP'/MBP'	25	Normal	24–36
mld/mld; MBP'/MBP'	27[c]	Normal	N.D.
shi/+	50	Normal	24–36
+/+	100	Normal	24–36
+/+, *MBP'/–*	112.5[c]	Normal	N.D.
+/+, *MBP'/MBP'*	125[c]	N.D.	N.D.

[a] mRNA levels in mutant and transgenic mice (Popko *et al.*, 1987).
[b] Experiment not done.
[c] mRNA levels were not measured in these animals; the numbers represent the theoretical expectation.
[d] No convulsions were seen in these animals, although very few animals were observed for a limited time.

had 20 and 30% of normal levels of MBP mRNA and showed a shivering phenotype. This is a surprising result since transgenic *shi/shi* mice which expressed MBP mRNA levels of 25% of normal no longer had a shivering phenotype. Part of the reason for this discrepancy may have to do with the abnormal spatial distribution of MBP in the CNS of the antisense transgenic mouse as shown by immunohistochemical staining of MBP (Katsuki *et al.*, 1988). A large part of the white matter did not stain, but the staining in the cerebellum was patchy. Certain localized regions of the CNS that lack MBP may lead to a shivering phenotype without an overall reduction of MBP. In the transgenic shiverer mouse, the brain and spinal cord showed the same pattern of staining with anti-MBP as the normal mouse, although staining was somewhat less intense due to the lower levels of MBP (H. David Shine, unpublished observations).

DOUBLE MUTANTS

Animal mutations on different chromosomes might be expected to have a phenotype resulting from the simple addition of two individual phenotypes. Double mutants, such as shiverer and jimpy (*shi/shi; Tajp/Y*), and myelin deficient and jimpy^{msd} (shi^{mld}/shi^{mld}; $Tajp^{msd}/Y$), however, have unexpected and puzzling phenotypes (Kerner and Carson, 1986; Billings-Gagliardi *et al.*, 1986, 1987, 1988). Shiverer, myelin deficient and jimpy mutant mice have distinct morphological and biochemical characteristics. In shiverer as described above, most of the gene encoding MBP is deleted, and as a result the mutant lacks MBP, the number of myelinated axons is reduced, and there is no major dense line (MDL). Oligodendrocytes have abnormal shapes with numerous microprocess extensiosn that may be associated with axons (Billings-Gagliardi *et al.*, 1986). The CNS of the myelin deficient mutant differs from that of shiverer in that at least 2% of the myelin sheaths have a MDL (Shen *et al.*, 1985). In the jimpy mutant (Sidman *et al.*, 1964), the mRNA encoding PLP is abnormally spliced (Nave *et al.*, 1986, 1987; Macklin *et al.*, 1987) and no immunodetectable PLP is present (Kerner and Carson, 1983). In the CNS, the number of oligodendrocytes is reduced (Knapp *et al.*, 1986). The number of myelin sheaths also is reduced, but occasionally clustered myelinated axoms are seen with myelin of normal structure and thickness (Hirano *et al.*, 1969; Privat *et al.*, 1972; Meier and Bischoff, 1975; Wolf *et al.*, 1983). Numerous lipid-filled "jimpy" cells are found (Billings-Gagliardi *et al.*, 1986).

The shiverer and jimpy double mutant (*shi/shi; Tajp/Y*) has MBP, PLP, and CNP levels intermediate between those of the two single mutants (Kerner and Carson, 1986). The morphology of the CNS shows characteristics of both single mutants but is somewhat more normal than either. CNS myelin resembles shiverer, except that some clusters of myelinated axons are found that are char-

acteristic of jimpy in that they have a MDL (Billings-Gagliardi *et al.*, 1986) (Table II). Since the gene encoding MBP in *shi/shi* mice is largely deleted (Roach *et al.*, 1983, 1985), producing no detectable MBP message (Roach *et al.*, 1983) and no MBP in brain homogenates via radioimmunoassay (Dupouey *et al.*, 1979; Kirschner and Ganser, 1980; Readhead *et al.*, 1987), it is surprising that the double mutant *shi/shi*; *Tajp/y* has detectable levels of MBP in the brain and myelin with a major dense line. An explanation of these results could be that a "silent" MBP gene is expressed when the jimpy mutation is present. Any additional genes having homology to MBP, however, would have been detected by Southern blot analysis using an MBP cDNA probe (Takahashi *et al.*, 1985). Perhaps the genotype of the double mutants needs to be confirmed by Southern analysis.

The *shi^{mld}/shi^{mld}; Tajp/Y* double mutant has quite unexpected characteristics: levels of MBP, PLP, and CNP were indistinguishable from those in *shi^{mld}/shi^{mld}* alone, and the amount of myelin observed morphologically was much less than in either single mutant. No MDL was observed, in contrast to small amounts found in the *shi^{mld}/shi^{mld}* or *Tajp^{mld}/Y*. "Jimpy cells," characterized by autophagic vacuoles, were numerous (Table II). Structural abnormalities of *shi^{mld}/shi^{mld}* oligodendrocytes were absent in the double mutant (Billings-Gagliardi *et*

Table II. Comparison of Phenotypes of Shiverer and Jimpy Double Mutant Mice[a]

Genotype	Genetic defect	Number of myelinated axons in optic nerve (%)	Number of myelin sheaths showing a major dense line (%)	Abnormal lipid-filled "jimpy" cells
shi/shi	Deletion of major portion of MBP gene[(1)], Chr 18[(2)]	25	0	Absent
Tajp/y	Point mutation which results in defective splicing of PLP mRNA[(3)], Chr X[(4)]	6	84	Present
shi/shi * *Tajp/y*		23	2–3	Absent
shi^{mld}/shi^{mld}	Tandem duplication and inversion of MBP gene, Chr 18[(5)]	18	2	Absent
Tajp^{msd}/y	Allelic to jp[(6)]	10	94	Present
shi^{mld}/shi^{mld} * *Tajp^{msd}/y*		7	0	Present

[a] Morphological data were taken from mutant mice of the same B6C3 hybrid stock (Billings-Gagliardi *et al.*, 1986, 1987, 1988). References as follows: (1) Roach *et al.* (1983, 1985); (2) Sidman *et al.* (1985); (3) Nave *et al.* (1986, 1987) and Macklin *et al.* (1987); (4) Phillips (1954) and Sidman *et al.* (1964); (5) Popko *et al.* (1987) and Okano *et al.* (1987); (6) Macklin *et al.* (1988).

al., 1987). The phenotypes of the double mutants are difficult to interpret. A hypothesis of multiple primary gene function has been proposed, in which DNA at a given locus functions both as a structural gene coding for a protein and as a "cytogene" directing cellular function (Billings-Gagliardi *et al.*, 1987). Although the mutations are on different chromosomes, the defective genes are expressed in the same cell, the oligodendrocyte. A simpler explanation might be that imbalances or defects in PLP, MBP, and other proteins affect the functioning of the entire cell in unpredictable ways, since these proteins have to act in concert to build the myelin sheath. This is especially true for proteins that have to interact physically. These two explanations, however, are not mutually exclusive and these results bear further investigation.

CONCLUSION

Structural defects of the shiverer and myelin deficient mutations have been elucidated, and the impact of low levels or no MBP on myelin sheath has been seen clearly in electron microscopic studies. Yet the physiological consequences of a poor myelin sheath—shivering behavior, tonic seizures, and early death—still are difficult to understand. Since mice with different levels of MBP could be generated (see Table I), some of these questions were addressed. Detailed morphometric studies have shown that increasing the MBP gene dosage increases the number of myelinated axons and myelin sheath thickness (Shine *et al.*, 1988). Further analyses should lead to a better understanding of the behavioral aspects of these mutant mice.

Clearly, the number of axons that are myelinated and the thickness and compaction of the sheath are important for survival of the animal. An interesting series of questions that can be addressed using molecular and transgenic techniques is to what extent the integrity of the myelin sheath is dependent on the shape and structure of myelin proteins, and how this, in turn, affects the behavior of the animal. We expect that some of these questions will be answered in the near-future.

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