MOLECULAR AND CELLULAR BIOLOGY, Nov. 1991, p. 5479–5486
0270-7306/91/115479-08$02.00/0

Vol. 11, No. 11

Transgenic Mouse Model for Central Nervous System Demyelination

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Received 2 May 1991/Accepted 6 August 1991

A common feature of demyelinating diseases such as multiple sclerosis in humans and experimental autoimmune encephalomyelitis in rodents is the marked elevation in the expression of the major histocompatibility complex (MHC) antigens in the involved sites. By specific targeting of a syngeneic MHC class I gene to oligodendrocytes, we have generated transgenic mice which not only exhibit severe involuntary tremors and develop tonic seizures but also show extensive demyelination in both the brain and the spinal cord. The fact that demyelination in these mice occurs in the absence of immune infiltration dismisses an autoimmune involvement but suggests that the MHC class I antigens play a direct role in inducing disease. Our findings lend support to the possibility that demyelinating diseases are induced by infectious agents such as viruses which can either directly activate MHC gene expression in oligodendroglia or indirectly activate expression through the release by reactive T cells of gamma interferon in the brain.

Among neurological disorders, demyelinating diseases have drawn much interest because of the frequency with which they occur and the disabilities they cause. This group of disorders is likely to arise either from damage to the oligodendrocytes that produce the myelin or from a direct assault on the various components of the myelin sheath.

By far the most common of the demyelinating diseases is multiple sclerosis (MS). Clinically, MS presents in the form of recurrent attacks with frequent pathologic involvement of the brain, spinal cord, and optic nerves (2). Studies involving families with multiple cases of MS have suggested the presence of a susceptibility gene with moderately strong linkage disequilibrium to the major histocompatibility complex (MHC) in chromosome 6 and weaker linkages to known markers in other chromosomes (36). Epidemiological investigations, including studies on monozygotic twins discordant for MS (6, 23), have provided strong support for viral infections playing a direct role. Indeed, retroviruses, coronaviruses, myxoviruses, paramyxoviruses, and poxviruses have all been implicated in MS in various studies (5, 8, 11, 14, 17). Virus infection of oligodendrocytes in the central nervous system (CNS) may induce the breakdown of myelin and, subsequently, a secondary inflammatory response against specific autoantigens. Alternatively, it may induce a primary autoimmune reaction against certain components of the infected oligodendrocyte or the myelin, leading to secondary demyelination. While the exact etiology of MS remains unknown, any mechanism to explain the development of this disease must take into account a combination of genetic susceptibility, viral infections, and autoimmune responses.

Our present understanding of the pathogenesis of MS depends to a great extent on the study of appropriate animal models. One of the most intensely scrutinized experimental systems involves rats that are infected by the murine coronavirus (a strain of mouse hepatitis virus type 4) and develop chronic demyelination (16, 39). Although the accuracy of this model is debatable, one common occurrence in both the rat demyelinating encephalomyelitis and human MS is the marked elevation of expression in the brain of the MHC antigens, both class I and class II (24, 31, 32). This change involves different cell types and is likely to be induced by gamma interferon, a known activator of MHC class I and class II genes (9, 34, 38). The observations that monoclonal antibodies against MHC antigens can inhibit experimental autoimmune encephalomyelitis in the rodent model (25) and treatment with gamma interferon exacerbates attacks in humans with MS (20, 21, 30) suggest that products of the MHC genes must in each case play a role in the development of disease.

To test this hypothesis, we have derived transgenic mice with an elevated expression of a syngeneic MHC class I antigen specifically in oligodendrocytes of the CNS. This approach is unique in that it allows the investigation of the role of a single gene in the development of disease in the context of a whole animal (13, 18, 35).

MATERIALS AND METHODS

Production of transgenic mice. Transgenic mice were produced as described by Brinster et al. (4). Approximately 200 to 500 copies of the myelin basic protein (MBP)-K^b chimeric gene contained within the *Pvu*II-*Sal*I DNA fragment were injected into fertilized ova at the single-cell stage. Mice were screened for the acquisition of novel MBP and K^b DNA sequences by analysis of DNA obtained from tail biopsies (28).

Nucleic acid analysis. DNA was isolated from tail biopsies by the proteinase K-sodium dodecyl sulfate extraction procedure described previously (3). Purified DNA (10 μg) was digested with the appropriate restriction enzymes, electrophoresed in a 1% agarose gel, transferred to nitrocellulose filters, and hybridized with a ^{32}P-labeled DNA probe. RNA was extracted by the guanidinium isothiocyanate-CsCl gradient technique. Comparison of steady-state K^b and MBP-K^b mRNA levels can be achieved by using the reverse transcriptase-polymerase chain reaction (RT-PCR) procedure (33). Total tissue RNA obtained by the standard guanidinium thiocyanate-CsCl method is incubated with reverse

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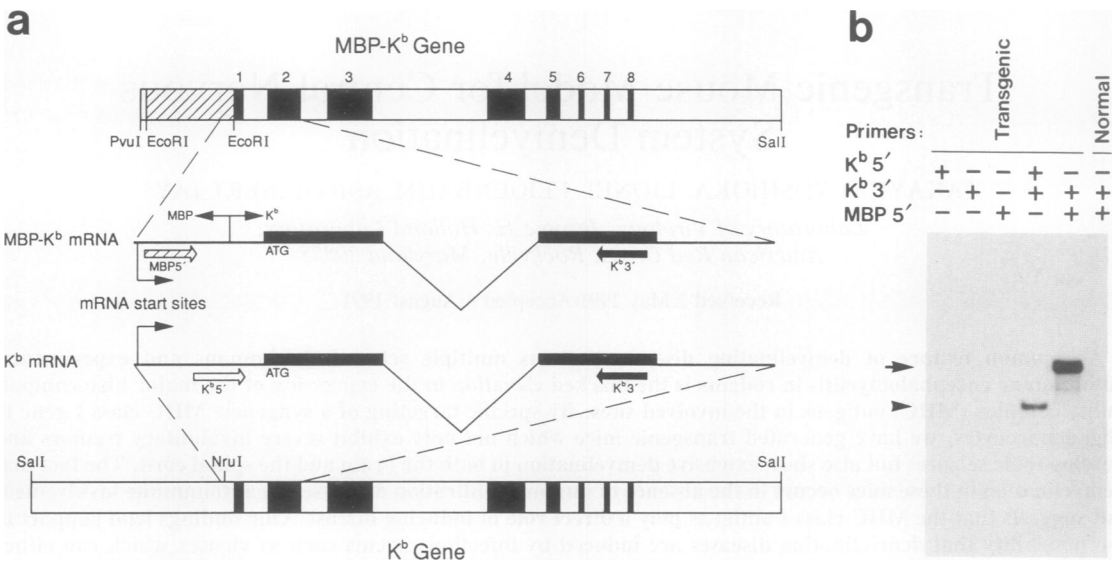


FIG. 1. Derivation of the MBP-K^b gene and detection of its transcriptional product. (a) The chimeric MBP-K^b gene. The wild-type class I K^b gene lying within a 9.5-kb *Sal*I DNA fragment (bottom) was digested with *Nru*I to remove all 5′ flanking sequences, including the transcriptional enhancer, promoter, RNA start site, and first 12 nucleotides of the mRNA (37). The remaining 5.5-kb segment contains all of the eight coding exons and 1.5-kb 3′ flanking sequences. The MBP regulatory region carrying the transcriptional enhancer, promoter, and RNA start site is contained within a DNA fragment bounded at the 5′ end by a *Hin*dIII site and at the 3′ end by a partial *Bbv*I site; the latter is located 36 nucleotides downstream of the RNA start site (12, 27). This 1.2-kb segment is ligated to the 5.5-kb K^b gene through *Eco*RI linkers to yield the MBP-K^b gene (top). This chimeric gene is expected to encode an mRNA which differs from the wild-type K^b transcript only in its 5′ noncoding region; the 5′-terminal 12 bases of the K^b mRNA are replaced by the 5′-terminal 36 bases of the MBP gene followed by an 8-base *Eco*RI linker sequence. The diagram is not drawn to scale. (b) The chimeric MBP-K^b transcript. Detection of expression of the MBP-K^b gene has to rely on its region of diversity from the wild-type K^b gene. Comparison of steady-state K^b and MBP-K^b mRNA levels can be achieved by using the RT-PCR procedure (33). Using RNA from the brain of a mouse carrying the MBP-K^b gene (transgenic), the 217-bp MBP-K^b PCR product (arrow) is detected only when both primers MBP5′ and K^{b}3′ are present. A similar component is not seen when RNA from a normal littermate not carrying the transgene (normal) is used. Separately, the 174-bp K^b PCR product (arrowhead) is detected only when both primers K^{b}5′ and K^{b}3′ are added. It is apparent that there is substantially more transgenic K^b than endogenous K^b mRNA in the brain of the transgenic mouse.

transcriptase in the presence of the common K^{b}3′ primer, a 19-mer (5′-dGTAGCCGACTTCCATGTAC-3′) that is homologous to the mRNA sequence within exon 2 of both the endogenous K^b and transgenic MBP-K^b genes. Amplification of the reverse transcriptase product is achieved by the separate addition of either primer K^{b}5′ (5′-dACTCAGA AGTCGCGAATCG-3′) or primer MBP5′ (5′-dCAGACCAT CCAAGAAGAAC-3′), each of which is unique to the wild-type or chimeric K^b cDNA, respectively. Each reaction is carried out in the presence of 100 ng of total RNA, 30 pmol of each appropriate primer, and 1 mmol of each deoxynucleoside triphosphate in 100 μl of reaction buffer (GeneAmp DNA amplification reagent kit; Perkin-Elmer Cetus). The mixture is heated at 68°C for 10 min and cooled to allow annealing. Avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) is then added at 5 U per reaction mixture. After incubation at 42°C for 10 min, *Taq* DNA polymerase (Ampli-Taq; Perkin-Elmer Cetus) is added at 1 U per reaction mixture, and the mixture is overlayered with a drop of mineral oil. DNA amplification is carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 20 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 1.5 min). To increase the sensitivity of detection of the PCR products, the amplified DNA fragments are subjected to liquid hybridization at 55°C using a ^{32}P-end-labeled oligonucleotide probe (5′-dCTCGCTGAGGTATTT CGTC-3′) that is homologous to a region located within exon 1 and detects both the K^b and MBP-K^b products.

Histological analysis. Tissues were fixed by incubation overnight with 10% formalin, embedded in paraffin, and stained either by hematoxylin and eosin or by Luxol fast blue (LFB). For electron microscopy, fixation was with paraformaldehyde and glutaraldehyde, and samples were embedded in Epon.

RESULTS

While several cell types, including astrocytes, vascular endothelial cells, and infiltrating B cells and macrophages, have been shown to express elevated levels of the MHC antigens within human MS lesions (24, 31, 32), a change in expression in oligodendrocytes which synthesize myelin in the CNS may be responsible for the ensuing demyelination.

To target the expression of the class I K^b gene selectively to oligodendrocytes, we have replaced the K^b gene regulatory sequence (37) with an analogous region from the gene for MBP (27) (Fig. 1). The resulting MBP-K^b gene will encode an mRNA which is indistinguishable from the wild-type K^b transcript except that the first 12 nucleotides have been replaced by 36 nucleotides from the 5′ noncoding region of the MBP gene. This chimeric transcript will encode an authentic K^b antigen and is not expected to have an altered turnover rate in the cell. While the transport of class I antigens to the cell surface requires their binding to β_2-microglobulin, previous transgenic and transfection stud-

ies have suggested the presence of an excess of β_2-microglobulin molecules in all cell types tested (29, 40).

To facilitate integration and efficient expression in target tissues, we excised the entire MBP-K^b transcriptional unit from all bacterial plasmid sequences by combined cleavage with *Pvu*I and *Sal*I (Fig. 1). The purified DNA fragment was microinjected into fertilized eggs from superovulated CD-1 females that were mated with C57BL/6 ($H\text{-}2^b$ haplotype) males (4). The CD-1 females were chosen because of the quality of their eggs, and the C57BL/6 males were used to provide a partial $H\text{-}2^b$ haplotype background from which the K^b gene was isolated.

Of the 24 mice born, 5 were found by Southern blot analysis to carry intact copies of the transgene. One of them, designated mouse C6, had severe tremors and occasional tonic seizures at a very young age, and died on day 28 postpartum. Its tissues were retained for further analysis. On breeding, two of the other transgenic mice, designated B5 and C1, were found to be either highly mosaic or unable to transmit the transgene. Much of the subsequent analysis was performed on mice from the two remaining founders, designated C2 and C7, which have been maintained as lines by backcrossing to C57BL/6 mice. Since the MHC on chromosome 17 of C57BL/6 and CD-1 mice can be distinguished by restriction fragment length polymorphism analysis using a cDNA probe derived from the 3′ noncoding region of the class I K^q gene (3, 28), founder animals were backcrossed to C57BL/6 mice, and offspring homozygous for the C57BL/6 MHC were selected for further breeding to generate established transgenic lines. As a result, the transgenic K^b gene is syngeneic with the $H\text{-}2^b$ background of these mice.

To demonstrate the specificity of the MBP regulatory region, we compared the tissue distribution of the endogenous K^b and the chimeric K^b transcripts in the two transgenic mouse strains by using quantitative RT-PCR analysis (33) (Fig. 1). Using a pair of primers, designated K^b5' and K^b3', which will detect selectively the endogenous wild-type K^b mRNA, a high level of accumulation was seen in thymus, lung, and liver and a somewhat lower level was seen in kidney and testis (Fig. 2a). The amount in brain and spinal cord was negligible. When the same RNA samples were analyzed with primers designated MBP5′ and K^b3', which specifically detect the transgenic K^b mRNA, a high level of accumulation was seen only in brain and spinal cord, and a low level was found in lung, liver, and testis (Fig. 2b). While RT-PCR analysis will provide only semiquantitative results, this approach is necessitated because Northern (RNA) blot analysis would not differentiate the endogenous K^b transcripts from transgene K^b transcripts.

From this analysis, it appears that the MBP transcriptional enhancer and promoter, shown to be specific for oligodendrocytes (12), is predominantly targeting the transgenic K^b gene to cells in the CNS, with only minor leakage of expression in certain other tissues. In addition, the presence of the transgene did not alter the pattern of expression of the endogenous K^b gene. The specificity of the MBP regulatory region is confirmed in both the C2 and C7 transgenic mouse lines as well as in the C6 founder mouse that died prematurely.

At about 2 to 3 weeks after birth, the MBP-K^b transgenic mice began to show physical signs of CNS involvement. Of the 22 F_1 mice from the C7 transgenic line that were studied, at least 18 showed a characteristic tremor (Fig. 3a). Virtually all of the mice also had tonic seizures, with rigid limbs and fully extended toes, that lasted less than 1 min (Fig. 3b). About half of these mice died at 4 to 5 weeks of age, and the

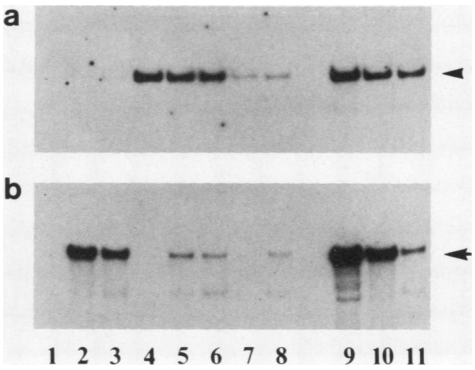
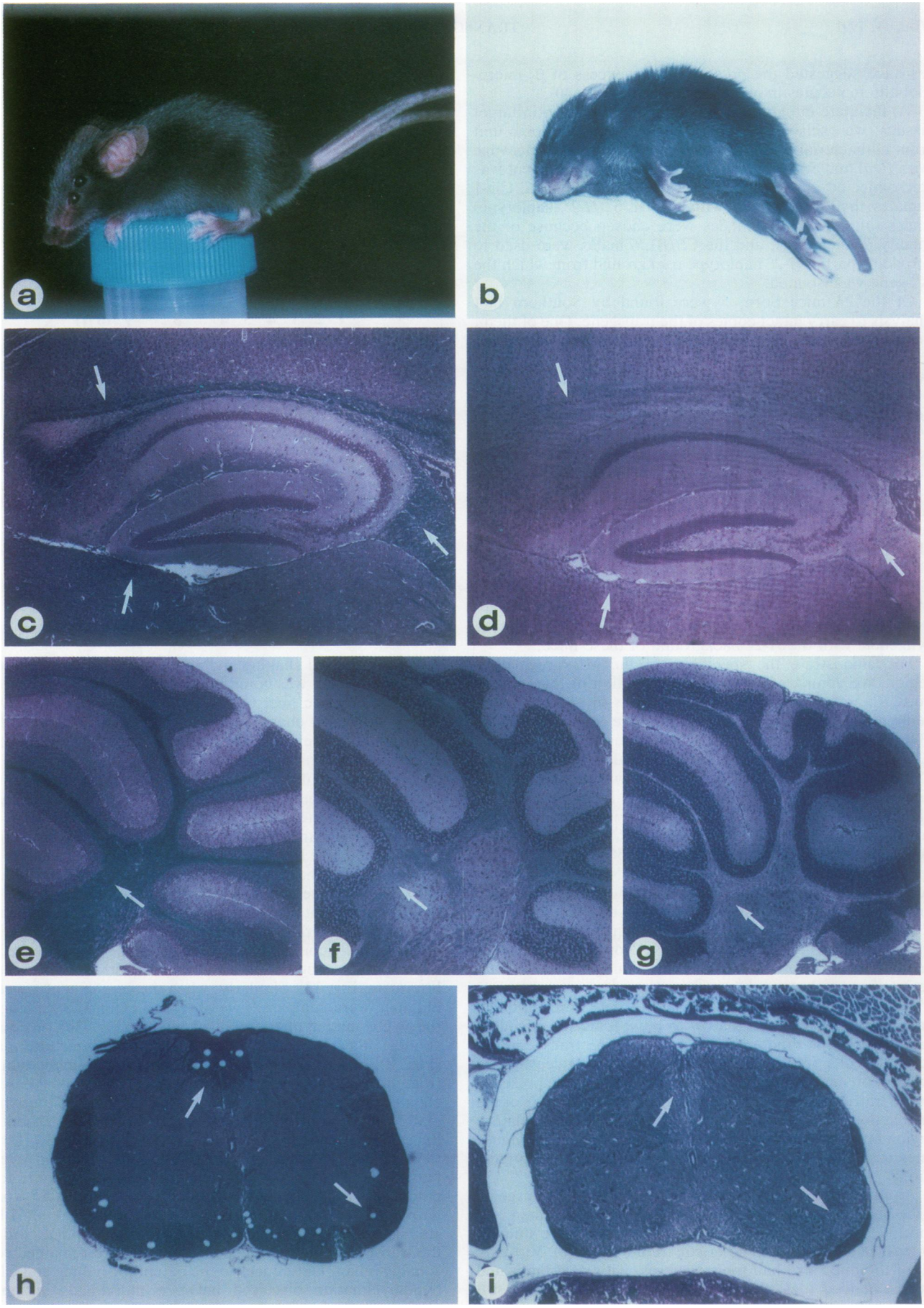


FIG. 2. Tissue distribution of the MBP-K^b transcript in transgenic mice. Total RNA was obtained from different tissues of a 3-week-old C7 mouse by the guanidinium-CsCl method. Total RNA (100 ng) was analyzed by the RT-PCR method. RNA samples used: none (lanes 1), brain (lanes 2), spinal cord (lanes 3), thymus (lanes 4), lung (lanes 5), liver (lanes 6), kidney (lanes 7), and testis (lanes 8). (a) Primers K^b5' and K^b3' were used to detect and quantitate the endogenous K^b transcript. A titration of liver RNA at 300 ng (lane 9), 100 ng (lane 10), and 33 ng (lane 11) was performed at the same time to demonstrate that the assay was RNA concentration dependent and quantitative. The position of the 174-bp PCR product is indicated (arrowhead). (b) Primers MBP5′ and K^b3' were used for detection of the transgenic K^b transcript. A titration of brain RNA at 300 ng (lane 9), 100 ng (lane 10), and 33 ng (lane 11) was also included. The position of the 217-bp PCR product is indicated (arrow).

remaining ceased shaking and appeared to have recovered. This observation was confirmed by examining F_1 mice from the C2 transgenic line. Of the 22 mice observed, no fewer that 8 showed a characteristic tremor and occasional episodes of tonic seizure. They appeared to have recovered from shaking.

The physical diagnosis and sites of expression of the transgene prompted a detailed analysis of the brains and spinal cords of these mice. Parallel comparisons of MBP-K^b transgenic mice and their normal littermates at different ages were carried out. Microscopic examination of formalin-fixed tissue sections that had been stained with LFB revealed a marked difference in myelin staining between the mice.

While abundant blue staining of myelin was detected in the hippocampus of the control mouse at 4 weeks of age (Fig. 3c), a virtually complete absence of staining was observed in a comparable region of the brain of the C7 transgenic littermate (Fig. 3d). Similarly, when the cerebella of 4-week-old mice were compared, we observed extensive staining in the white matter of the control mouse (Fig. 3e), marginal staining in a comparable region of a C2 transgenic littermate (Fig. 3f), and virtually no staining in a C7 transgenic mouse (Fig. 3g). The difference in staining within the spinal cord is equally impressive. The blue staining throughout the white matter of the control mouse (Fig. 3h) was totally absent in the sample from the C7 transgenic littermate (Fig. 3i). It has to be pointed out that LFB is not a specific stain for myelin but instead detects lipids; this lack of staining by LFB does not necessarily mean the absence of myelin. It is worth noting, however, that despite the apparent lack of LFB staining in different regions of the brain and spinal cord of the transgenic mice, the cellular architecture remained reasonably intact. Differences in the extent of shaking and CNS hypomyelination observed between individuals within a transgenic strain or between strains may be explained by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either differences in the level of expression of the transgene, differences in the developmental onset of expression of the transgene, or differences in the genetic background of the individual mouse.

To better visualize the hypomyelination at the level of individual axons, electron microscopic analysis of thin sections from the optic nerves of both young and old mice was performed. While normal myelin sheath thickness is observed in axons of a 3-week-old control mouse (Fig. 4a), myelin sheaths with no more than half the normal thickness are seen in the C7 transgenic littermate (Fig. 4b). In addition, apparently empty spaces are found scattered throughout. On careful examination, outlines or remnants of myelinated axons are seen in these spaces (Fig. 4b, arrowheads), together with subcellular organelles found extracellularly. This may suggest active demyelination and possible axonal death rather than just hypomyelination.

To explain the apparent recovery from the shaking and tonic seizures with increasing age, we analyzed littermates at 4 months of age. While the control mouse showed a thick myelin sheath around every axon (Fig. 4c), the transgenic littermate had much thinner wrappings around the individual axons (Fig. 4d). In contrast to younger mice, there were no apparent void spaces that are indicative of active demyelination. When the sciatic nerves from control and transgenic littermates were similarly compared (data not shown), there was no sign of demyelination in the peripheral nervous system.

Could the hypomyelination result from down-regulation of expression of the MBP gene by the transgenic MBP-K^b gene? Total brain RNA from control and transgenic littermates was isolated at 3, 4, and 5 weeks after birth and subjected to RT-PCR analysis using the same MBP5′ primer that was used for the quantitation of the MBP-K^b transcript, in conjunction with the MBP3′ primer which was derived from exon 2 of the MBP gene. No reduction in the level of MBP mRNA was observed in the transgenic mice (Fig. 5a, lanes 4 to 6) in comparison with the control littermates (lanes 1 to 3). In fact, there was a slight and reproducible elevation in the amount of MBP mRNA. This finding suggests that expression of the MBP-K^b gene in no way affected the transcription of the endogenous MBP gene.

Western immunoblot analysis of MBP in the brain revealed a significant decrease in the accumulation of MBP in a 3-week-old C7 transgenic mouse (Fig. 5b, lane 2) in comparison with its normal littermate (lane 1). However, the 5-month-old C7 transgenic mouse (lane 4) had approximately half the level of MBP found in its normal littermate (lane 3). These observations confirm the ultrastructural studies which demonstrate extensive demyelination at 3 weeks and significant hypomyelination at 4 months of age.

DISCUSSION

Expression of the MBP gene begins at about day 5 after birth, gradually increases until day 18, and then stabilizes at roughly one-fourth the peak level throughout adult life (42). This pattern of developmental regulation is determined by interactions between cellular *trans*-acting factors and *cis*-acting elements within the MBP control region. The phenotype that we observed in our mice is consistent with the expression of the K^b transgene under the control of the MBP regulatory sequence. At 2 to 3 weeks of age, when the MBP promoter is most active, the mice began to have involuntary tremors and develop tonic extensor seizures. Within 1 to 2 weeks, when the MBP promoter activity declines by three-fourths, the mice appear to recover. At the ultrastructural level, extensive demyelination and apparent axonal death were detected also at 2 to 3 weeks but not at later times. It appears that a certain threshold level of the MHC class I antigen is required to induce demyelination.

Since oligodendrocytes in culture can be activated to express endogenous class I genes (26), it appears that this set of genes may be actively suppressed in the brain. The phenotypic changes that we observed in the MBP-K^b transgenic mice are most likely not the result of the transgene interfering with expression of essential developmental genes, including the competition for transcription factors, since MBP RNA accumulation was not decreased.

While hypomyelination is clearly observed in our transgenic mice, there is also morphologic and biochemical evidence for demyelination early on after birth when the MBP regulatory elements are transcriptionally most active. This process, however, does not seem to be accompanied by any obvious signs of immune infiltration, suggesting that the demyelination observed is not of autoimmune etiology. It is therefore tempting to hypothesize that the presence of autoreactive T cells or B cells is not a prerequisite for active demyelination. One might speculate that an elevated level of MHC class I antigens, which function on the cell surface as adhesion molecules, may interfere with either the membrane targeting of specific myelin components or the compaction of the myelin sheath. Such an alteration may suffice to induce autodegradation and demyelination. It is interesting to note that the extremely high rate of sustained synthesis of myelin in the CNS (19) may reflect a process of autodegradation and replacement even in normal individuals.

While immune infiltration is apparently not the initiating event in the demyelination that we observed, its presence could add substantially to the development of a full-blown disease (1, 41). The fact that a secondary inflammatory response was not observed in the MBP-K^b mice may be due to the fact that C57BL/6 mice appear to be resistant to experimental autoimmune encephalomyelitis (7, 15). We suspect that with the extent of demyelination observed in the MBP-K^b mice, an autoimmune response against certain components of myelin could occur if the mice were of a susceptible genetic background. If autoimmunity is induced, then secondary demyelination could occur and worsen the disease.

FIG. 3. Gross examination and microscopic analysis of control and transgenic mice. (a) A 16-day-old C7 transgenic mouse with a severe tremor. (b) A 21-day-old C2 transgenic mouse during an episode of tonic seizure. (c) LFB-stained section of the hippocampus of a 4-week-old control mouse. Areas of intense staining are indicated (arrows). (d) LFB-stained section of the hippocampus of a 4-week-old C7 transgenic littermate. Areas where staining are normally detected are indicated (arrows). (e) LFB-stained section of the cerebellum of a 4-week-old control mouse, with intense staining in the white matter (arrow). (f) LFB-stained section of the cerebellum of a 4-week-old C2 transgenic mouse, with weak staining in the white matter (arrow). (g) LFB-stained section of the cerebellum of a 4-week-old C7 transgenic mouse, with no staining in the white matter (arrow). (h) LFB-stained section of the spinal cord of a 4-week-old control mouse, with intense staining in the white matter (arrows). (i) LFB-stained section of the spinal cord of a 4-week-old C7 transgenic mouse, with no staining in the white matter (arrows).

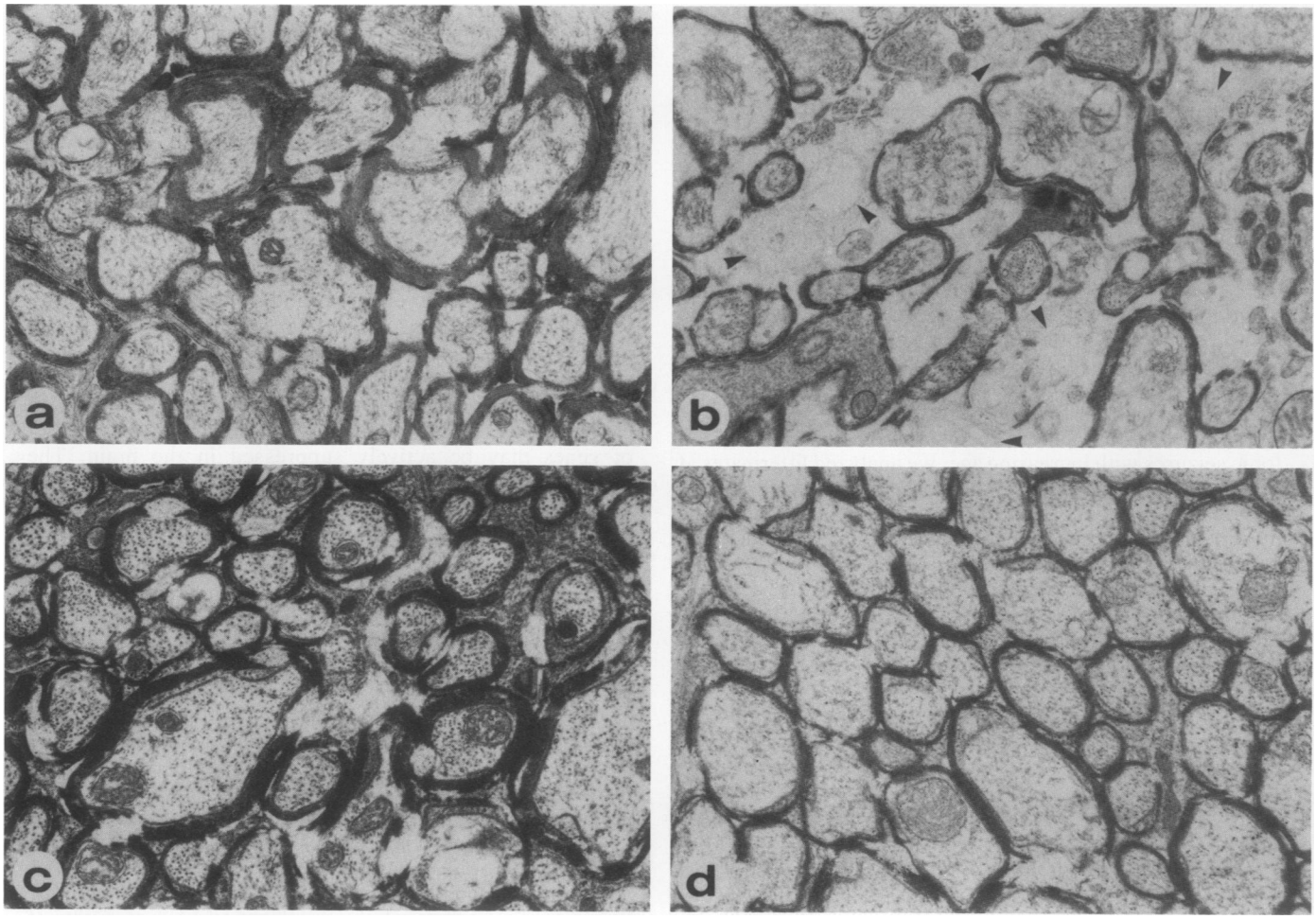

FIG. 4. Electron micrographs of sections of the optic nerve from control and transgenic mice. (a) A 24-day-old control mouse; (b) a 24-day-old C2 transgenic littermate. Areas showing remnants of myelinated axons are indicated (arrowheads). (c) A 16-week-old control mouse; (d) a 16-week-old C2 transgenic littermate. Magnification, ×5,183.

The result of these experiments may explain why such a large number of human viruses have been implicated in the pathogenesis of MS (5, 8, 11, 14, 17). The inability in the past to associate a single infectious agent with the development of disease has been disturbing because of the suggestion of lack of specificity. We propose that it is the induction of MHC antigens which leads to demyelination. Accordingly, any neurotropic virus could contribute to the onset of disease. In fact, several viruses, including human T-lymphotropic virus type I, which has been speculated as the causative agent of MS (14), have already been shown to induce class I gene expression in cells in culture (22). The upregulation in vivo can be achieved either directly as a consequence of viral infection of oligodendroglia or indirectly as a result of gamma interferon production by reactive T cells which in turn induce MHC expression in oligodendroglia (9, 34). Furthermore, any local inflammatory response, not necessarily involving virus infection, may also lead to the release of gamma interferon and to the same clinical endpoint. This view is strongly supported by observations that treatment of MS patients with gamma interferon markedly exacerbated the disease (20, 21, 30).

Our findings with the MBP-K^b transgenic mice not only demonstrated the direct involvement of MHC class I gene expression in oligodendrocytes in initiating the process of CNS demyelination but also allowed the speculation of how severe infections or even operative traumas may each be responsible for indirectly inducing MHC gene expression and subsequent demyelination. Since MHC genes are highly polymorphic, individual alleles having been shown to function differently perhaps as a consequence of variation in ligand binding affinities (10), it may not be surprising that differences in genetic susceptibilities exist in the development of MS in humans (27).

The transgenic mouse model presented here suggests that if a certain level of MHC class I antigen is not sustained, partial remyelination may ensue and recovery can be achieved. This may be analogous to an episode of attack and remission in early stages of human MS (2). We suspect that downward progression to permanent deficit, after repeated attacks, must involve a chronic secondary inflammatory response against specific myelin components (1, 41). Therefore, it will be important for us to test this possibility by backcrossing our C57BL/6 transgenic mice to SJL mice, which are highly susceptible to experimental autoimmune encephalomyelitis (7, 15). It will also be interesting to determine whether expression of MHC class II antigens in oligodendrocytes might also lead to CNS demyelination.

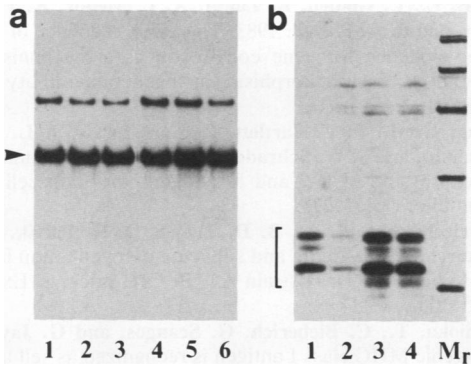

FIG. 5. Expression of the MBP gene in control and transgenic mice. (a) RT-PCR analysis using primers MBP5′ and MBP3′ of total brain RNA from either control mice (lanes 1 through 3) or C7 transgenic littermates (lanes 4 through 6) that were sacrificed at 3 weeks (lanes 1 and 4), 4 weeks (lanes 2 and 5), and 5 weeks (lanes 3 and 6) of age. The position of the 238-bp RT-PCR product is indicated (arrowhead). (b) Western immunoblot analysis using a rabbit anti-MBP serum of total brain extracts from either control mice (lanes 1 and 3) or transgenic littermates (lanes 2 and 4) that were sacrificed at 3 weeks (lanes 1 and 2) and 20 weeks (lanes 3 and 4) of age. Positions of the abundant MBP components are indicated (bracket). The molecular weight markers (M_r) used were 92,000, 68,000, 45,000, 30,000, and 12,000.

ACKNOWLEDGMENTS

We are grateful to Motoya Katsuki for the gift of the MBP antibody. We thank Jonathan Vogel, Charles Bieberich, and Frank LaFerla for critical reading of the manuscript, and we thank Lisa Ruiz and Lucie Rainone for help in preparation of the manuscript.

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