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Targeted Deletion of the Antisilencer/Enhancer (ASE) Element from Intron 1 of the Myelin Proteolipid Protein Gene (*Plp1*) in Mouse Reveals that the Element Is Dispensable for *Plp1* Expression in Brain during Development and Remyelination

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Abstract

Myelin proteolipid protein gene (*Plp1*) expression is temporally regulated in brain, which peaks during the active myelination period of CNS development. Previous studies with *Plp1-lacZ* transgenic mice demonstrated that (mouse) *Plp1* intron 1 DNA is required for high levels of expression in oligodendrocytes. Deletion-transfection analysis revealed the intron contains a single positive regulatory element operative in the N20.1 oligodendroglial cell line, which was named ASE (antisilencer/enhancer) based on its functional properties in these cells. To investigate the role of the ASE *in vivo*, the element was deleted from the native gene in mouse using a Cre/*lox* strategy. While removal of the ASE from *Plp1-lacZ* constructs profoundly decreased expression in transfected oligodendroglial cell lines (N20.1 and Oli-neu), the element was dispensable to achieve normal levels of *Plp1* gene expression in mouse during development (except perhaps at postnatal day 15) and throughout the remyelination period following cuprizone-induced (acute) demyelination. Thus, it is possible that the ASE is nonfunctional *in vivo*, or that loss of the ASE from the native gene in mouse can be compensated for by the presence of other regulatory elements within the *Plp1* gene.

Keywords

cuprizone demyelination; enhancer; gene regulation; gene targeting; myelin proteolipid protein; remyelination

INTRODUCTION

The myelin proteolipid protein gene (*Plp1*) encodes the most abundant protein found in mature myelin from the CNS (Eng *et al*. 1968; Norton *et al*. 1973). Two major gene products are generated via alternative splicing. The DM20 isoform is identical to PLP except it lacks an internal 35 amino acid stretch encoded by the distal portion of exon 3 (Macklin *et al*. 1987; Nave *et al*. 1987b; Simons *et al*. 1987). PLP has been proposed to function as an

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adhesive strut between apposing layers of the myelin sheath, aiding in the compaction of CNS myelin and conferring structural stability (Gow *et al.* 1997). However, more recently, additional functions have been ascribed for PLP/DM20 including roles in oligodendrocyte-neuron interactions (Yool *et al.* 2001), signaling (Gudz *et al.* 2003), and programmed cell death (Skoff *et al.* 2004; Hüttemann *et al.* 2009).

The gene is present in a single copy on the X chromosome, and as such, several X-linked recessive disorders – Pelizaeus-Merzbacher disease (PMD) and spastic paraplegia type 2 (SPG2) – arise from mutations in the human *PLP1* gene (for reviews see Inoue 2005; Garbern 2007). These dysmyelinating disorders can be caused by a lack of the protein due to deletion of the chromosomal region containing *PLP1*, or conversely from increased *PLP1* gene dosage, resulting in *PLP1* overexpression and oligodendrocyte dysfunction. Thus, *PLP1* gene activity in oligodendrocytes must be tightly regulated, befitting a 'Goldilocks scenario' where too little or too much of the protein have negative consequences. Expression of the gene is temporally regulated in oligodendrocytes (reviewed in Wight and Dobretsova 2004), with peak levels being attained during the active myelinating period of CNS development.

Previous studies with mice that harbor the PLP(+)Z transgene [contains mouse *Plp1* genomic DNA from the proximal 2.4 kb of 5′-flanking DNA to the first 37 bp of exon 2 linked in-frame with a *lacZ* reporter gene] demonstrated that the *Plp1-lacZ* fusion gene was expressed in brain, in a temporal manner, consistent to that of the endogenous *Plp1* gene (Wight *et al.* 1993; Li *et al.* 2002b). Yet expression of a comparable transgene, PLP(−)Z, which altogether lacks *Plp1* intron 1, was very much blunted, resulting in low levels of *Plp1-lacZ* gene activity in brain throughout development (Li *et al.* 2002b). Taken together, these results suggest that *Plp1* intron DNA contain regulatory element(s) that are important in mediating the dramatic increase in *Plp1* gene activity observed during the active myelination period. Subsequently, deletion-transfection analysis was performed with *Plp1-lacZ* constructs that lack a portion of *Plp1* intron 1 DNA to map the location of these regulatory element(s). Several regulatory elements were found to be functionally active in N20.1 cells [immortalized oligodendroglial cell line derived from mouse which expresses the *Plp1* gene (Verity *et al.* 1993)], consisting of a couple of negative regulatory elements (Dobretsova and Wight 1999; Li *et al.* 2002a) and a single positive element (Dobretsova and Wight 1999). The positive element, termed ASE for antisilencer/enhancer, is located within intron 1 positions 1083-1177 (Dobretsova *et al.* 2000), based on numbering the entire intron from positions 1 to 8140 (Wight and Dobretsova 1997). To test whether the ASE is the element responsible for mediating the surge in *Plp1* gene activity during the active myelination period of CNS development, it was targeted for deletion from the native gene in mouse through homologous recombination, using a Cre/*lox* system. Results presented here suggest that the ASE is largely dispensable for the developmental regulation of *Plp1* gene expression *in vivo*. Likewise, lack of the ASE did not seem to impair *Plp1* gene expression in adult mice actively undergoing CNS remyelination.

METHODS

Cell culture

Two mouse oligodendroglial cell lines were used in the study. N20.1 cells were grown at 34°C in a 1:1 mixture of Ham's F-12/Dulbecco's modified Eagle's low-glucose medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15mM HEPES, 2.438 g/L sodium bicarbonate, 4 g/L glucose, 100 μg/mL G-418, 10% fetal bovine serum (HyClone, Logan, UT, USA), and maintained in an atmosphere of 5% CO_2. Oli-neu cells (Jung *et al.* 1995) were grown at 37°C in SATO medium devoid of mitogens (ODM) according to the

modifications by He *et al.* (2007), and supplemented with 1% horse serum (Invitrogen). Oli-neu cells were maintained in an atmosphere of 10% CO_2.

Plp1-lacZ constructs

Details regarding the generation of *Plp1-lacZ* constructs, PLP(+)Z (Wight *et al.* 1993), PLP(−)Z (Wight and Dobretsova 1997), PLPΔ809-5807 (Dobretsova and Wight 1999), and PLPΔ809-5807 F(−AP4) (Dobretsova *et al.* 2004), referred to here as 'PLPΔ809-5807 + ASE', has been reported, previously. These constructs possess mouse *Plp1* genomic DNA [proximal 2.4 kb of 5′-flanking DNA, all of exon 1 DNA, and the first 37 bases of exon 2] which is used to drive expression of a *lacZ* reporter gene cassette. The constructs differ only in the amount of *Plp1* intron 1 DNA included; PLP(+)Z contains the entire intron (8140 bp), whereas PLP(−)Z totally lacks it. The PLPΔ809-5807 construct is missing intron 1 positions 809 to 5807, including the ASE sequence, based on numbering *Plp1* intron DNA from positions 1 to 8140 (Wight and Dobretsova 1997. The ASE sequence (intron 1 positions 1093-1177) was reinstated at the deletion-junction site of PLPΔ809-5807 to generate the PLPΔ809-5807 + ASE construct.

The *Plp1-lacZ* construct, PLPΔASE, was produced with a PCR fragment (2214 bp) generated from genomic DNA of $Plp1^{\Delta ASE}$ mice (described below), and encompasses a region of *Plp1* intron 1 in which the ASE sequence has been deleted. *Plp1* sense (ΔASE-For; 5′-AGGAGTTCAACTTTGGGGCTTTG-3′) and antisense (ΔASE-Rev; 5′-AAAGCTCGAGGAACCAGGTGTC-3′) primers were utilized for PCR. The resulting amplicon was digested with *San*DI and *Nhe*I, and exchanged for the corresponding region in PLP(+)Z. Hence, PLPΔASE is identical to PLP(+)Z except for missing 93 bp of *Plp1* intron 1 DNA, and contains a *lox*P site in lieu of the ASE.

Transfection analysis

Methodology for transient transfection analysis in N20.1 cells using Lipofectamine Transfection Reagent (Invitrogen) has been described, previously (Dobretsova and Wight 1999). Briefly, N20.1 were seeded at a density of 2.8×10^5 cells in 2 mL of growth medium per 35-mm well (6-well plate; Costar, Cambridge, MA, USA). The following day, cells were treated with a mixture of 5 μL of Lipofectamine and 2 μg of total DNA per well. Wells contained equimolar amounts of a specific *Plp1-lacZ* construct plus 0.35 μg of RSVL to control for differences in transfection efficiency; the RSVL plasmid utilizes the RSV promoter to drive expression of a luciferase reporter gene (de Wet *et al.* 1987). Empty vector (pBluescript SK+; Stratagene, La Jolla, CA) was used either alone to determine the background with the reporter gene assays, or as filler to keep the total amount of DNA transfected constant. Cells were incubated for 6 h with the Lipofectamine/DNA mixture and lysates prepared approximately 72 h post-DNA addition in 190 μL of Reporter Lysis Buffer (Promega, Madison, WI, USA) as recommended by the supplier.

Oli-neu cells were seeded at 2.5×10^5 cells per 35-mm well in 2 mL of growth medium the day prior to transfection. The FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN, USA) was used to transfect the cells according to manufacturer's specifications. The cells within a given well were treated with a mixture of FuGENE 6 (4.5 μL) and 3 μg of total DNA, which contained the same amount of *Plp1-lacZ* test construct and RSVL as used for N20.1 cells, with slightly more pBluescript SK+ to bring the total amount of DNA to 3 μg. The cells were left undisturbed for 48 h post-DNA addition, after which cell lysates were prepared as described for N20.1 cells.

Cell lysates (10-μl aliquots) were evaluated for reporter gene expression (in triplicate) using the Galacto-Light Plus Kit (Tropix, Bedford, MA, USA) and Luciferase Assay System

(Promega) for determination of β-galactosidase (β-gal) and luciferase activity as described, previously (Dobretsova and Wight 1999). Luminescence was measured as relative light units (RLU) in an AutoLumat LB 953 luminometer (EG&G Berthold, Gaithersburg, MD, USA). Transfection results represent the mean ± SD of β-gal activity relative to that obtained for PLP(−)Z transfected cells, which was arbitrarily set at 100% (N20.1 cells) or 1-fold (Oli-neu cells) in every experiment. Results were compiled from three or more independent experiments.

Construction of the *Plp1*^{NeoΔASE} targeting vector and associated plasmids

The plasmid, pOSDUPDEL [gift from Dr. Oliver Smithies, University of North Carolina, Chapel Hill], which contains a floxed (flanked by *lox*P sites) neomycin resistance (Neo) gene cassette from pMC1Neo (Thomas and Capecchi 1987) for positive selection and a herpes simplex virus (HSV)-thymidine kinase (TK) gene cassette driven by the mouse phosphoglycerate kinase (*Pgk*) promoter for negative selection, was used to generate the *Plp1* targeting vector. The *Plp1*-specific sequences (regions of homology) in the targeting vector were obtained by PCR using mouse genomic DNA (129/SvJ) as the template. The 5′ homologous region (‘left arm’) includes *Plp1* DNA from the proximal 2.4 kb of 5′-flanking DNA, downstream, to intron 1 position 1083. Approximately 200 ng of genomic DNA was amplified by PCR for 35 cycles [denaturation at 94°C for 30 s, annealing at 52.2°C for 30 s, and extension at 68°C for 230 s] using the *Age*I sense (5′-CACCGGTCACGTTTTCTGGTTTACTGCTAGAACTG-3′) and *Xho*I antisense (5′-ACTCGAGCCAAACCTGGTTGTTTGGACCAGT-3′) primers which incorporate convenient restriction enzyme sites (underlined sequence) for cloning. The PCR-generated DNA was digested with *Age*I and *Xho*I and the 3.7-kb fragment cloned immediately upstream of the Neo cassette in pOSDUPDEL in a like orientation. The resulting plasmid was designated ‘Clone A’. The 3′ homologous region (‘right arm’) contains *Plp1* genomic DNA extending from intron 1 position 1177, downstream, to the first 37 bp of exon 2. As well, genomic DNA encompassing another region of *Plp1* was amplified by PCR for 35 cycles [denaturation at 94°C for 30 s, annealing at 57.6°C for 30 s, and extension at 68°C for 7.5 min] using *Pac*I sense (5′-GTTAATTAATTTTGTCTACTTTCAGGGTGGAAGATCAAGTAATAAGGATGTGGC-3′) and *Not*I antisense (5′-TGCGGCCGCCCCCTACCAGACATCTAGCACAACACT-3′) primers. The PCR product was digested with *Pac*I and *Not*I restriction enzymes and the resulting 7.0-kb fragment inserted in Clone A immediately downstream of the Neo gene cassette to generate the *Plp1*^{NeoΔASE} targeting vector (Clone B). The sequence for both arms of homology and the Neo gene cassette were verified by DNA sequencing. Hence, the successfully targeted *Plp1* gene will contain the Neo gene cassette in lieu of the ASE sequence [i.e., missing *Plp1* intron 1 DNA positions 1084-1176].

Clone P was generated to screen for homologous recombination between the targeting vector and the native *Plp1* gene in embryonic stem (ES) cells (i.e., contains the *Plp1*^{NeoΔASE} allele). To aid in the construction of Clone P, another plasmid was generated, called CK-7. CK-7 possesses a *Cla*I-*Kpn*I fragment (~7 kb) of mouse *Plp1* genomic DNA [5′-flanking DNA to the proximal third of intron 1] isolated from clone KK1 (Macklin *et al.* 1991) and inserted into the analogous sites in the pGEM-7Zf(−) vector (Promega). Clone A and CK-7 were digested with *Aat*II and *Ngo*M IV and the resulting 3.2-kb *Aat*II-*Ngo*M IV fragment from Clone A was ligated to the 5.1-kb fragment from CK-7 [includes pGEM-7Zf(−) vector backbone] to generate Clone P. Clone P contains *Plp1* genomic DNA [proximal 3.6 kb of 5′-flanking DNA to intron 1 position 1083] fused to the 5′ portion of the Neo gene cassette.

Gene targeting and generation of knock in mouse lines

Pluripotent ES cells (W4) were electroporated with the targeting vector (*Not*I-linearized) at the Gene Targeting Facility (University of Iowa, Iowa City), and subsequently grown in the presence of G-418 and ganciclovir for positive/negative selection, respectively. The resulting ES colonies were isolated, expanded, and screened for homologous recombination. Initially, clones were screened for homologous recombination with the left arm of the targeting vector by PCR using a *Plp1* sense (P1; 5′-ACTTCTCAATGCCCTGGTTCAGTGGAGC-3′) and Neo antisense (P1′; 5′-CGTCACCTTAATATGCGAAGTGGACCTGG-3′) primer pair which results in a 4.1-kb amplicon. PCR conditions were: denaturation at 94°C for 2 min, followed by 40 cycles of amplification [denaturation at 94°C for 30 s, annealing at 58.5°C for 30 s, extension at 68°C for 246 s], and final extension at 68°C for 5 min. The PCR products were fractionated by gel electrophoresis on a 0.8% agarose gel and visualized by staining with ethidium bromide. Subsequently, ES clones deemed positive in the initial (PCR) analysis were evaluated for homologous recombination at both ends through Southern blot analysis using standard techniques (Sambrook and Russell 2001). Briefly, genomic DNA (10 μg) from the ES clones was digested with either *Sph*I or *Ase*I for 3 h, fractionated on a 0.7% agarose gel, and transferred to a nitrocellulose membrane using 10× SSC. Homologous recombination with the left arm of the targeting vector was assessed using a 4-kb ^{32}P-radiolabeled probe (*Bam*HI fragment of mouse *Plp1* genomic DNA located 1.4 kb upstream of exon 1). Homologous recombination with the right arm of the targeting vector was evaluated by hybridization to a 3-kb ^{32}P-labeled probe prepared from an *Nde*I fragment of *Plp1* intron 1 DNA (see Fig. 2a for further details). The blots were washed 3-4 times in a solution of 2× SSC and 0.1% SDS at 22°C for 1 h, and then twice in a solution of 0.1× SSC and 0.1% SDS at 42°C for 2 h. Following the washes, the blots were subjected to autoradiography. Hybridization of the *Bam*HI probe with *Sph*I digested genomic DNA results in 17.6-kb band from the native (wild-type; WT) *Plp1* gene or a 7.7-kb band from the rearranged gene (*Plp1*$^{\text{Neo}\Delta\text{ASE}}$). Likewise, hybridization to *Ase*I digested DNA results in a 14.6 kb-band for the WT allele and an 8.6 kb-band for *Plp1*$^{\text{Neo}\Delta\text{ASE}}$ [7.4-kb band for *Plp1*$^{\Delta\text{ASE}}$ after excision the Neo cassette with Cre recombinase]. Hybridization of the *Nde*I probe to *Ase*I cleaved DNA results in a 14.6-kb band for the WT gene and a 7.1-kb band with the rearranged allele (*Plp1*$^{\text{Neo}\Delta\text{ASE}}$).

W4/129S6 ES clones ES901 and ES914, which contain the *Plp1*$^{\text{Neo}\Delta\text{ASE}}$ rearrangement, were independently injected into C57BL/6J blastocysts and transplanted into pseudopregnant ICR females (Gene Targeting Facility, University of Iowa, Iowa City). [All procedures involving the use of mice were approved by the Institutional Animal Care and Use Committees at the University of Arkansas for Medical Sciences and the University of Iowa, in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Research Council's Guide for the Care and Use of Laboratory Animals, and adhered to ARRIVE guidelines (Kilkenny *et al.* 2010).] The resulting chimeras were bred to C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) to generate female heterozygotes (*Plp1*$^{+/\text{Neo}\Delta\text{ASE}}$). Because the *Plp1* gene is located on the X chromosome and the W4 ES cells are genotypically male, only female progeny will carry the rearranged allele. Germline transmission was assessed by PCR and Southern blot analyses similar to that described for the ES clones. Removal of the Neo gene cassette was achieved by breeding *Plp1*$^{\text{Neo}\Delta\text{ASE}}$ mice with EIIa-*Cre* transgenic mice (The Jackson Laboratory), which expresses Cre recombinase under control of the adenovirus EIIa promoter early in embryogenesis (Lakso *et al.* 1996). Genomic DNA was isolated from tail biopsies (0.5 cm) of mice, and genotyping performed using sense (WT2; 5′-GGGCTGCCACATCCTTATTA-3′) and antisense (WT2′; 5′-TGTGTTCACCGTGCAACTTT-3′) primers. The amplicon from the WT (*Plp1*) allele is

333 bp, while a 315 bp product is generated from the rearranged allele without Neo (*Plp1*$^{\Delta ASE}$); the *Plp1*$^{\Delta ASE}$ allele contains a single *lox*P site in lieu of the ASE. Failure to excise the Neo gene cassette from the *Plp1*$^{Neo\Delta ASE}$ allele would result in a 1505 bp amplicon.

RNA extraction

Mice were anesthetized with isoflurane, decapitated, and brains rapidly removed. Total RNA was isolated immediately thereafter from the brains, separately, using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA was resuspended in DEPC treated water, and samples aliquoted and stored at −70°C until further use.

Ribonuclease protection assay (RPA)

RPA analysis was performed as described previously (Li *et al*. 2002b) with the following modifications. To generate a plasmid from which to synthesize the *Plp1* riboprobe, *Plp1* sequence from the mouse cDNA, pRT5-7 (LeVine *et al*. 1990), was amplified by PCR and cloned into the *Bam*HI and *Eco*RI sites of pGEM-7Zf(−) using an exon 2 sense (5′-CGGGATCCTGTTAGAGTGTTGTGCTAGA TG-3′; incorporated *Bam*HI site underlined) and exon 3A antisense (5′-GGAATT CAGATGGTGGTCTTGTAGTCG-3′; incorporated *Eco*RI site underlined) primer pair. PCR conditions included 40 cycles of amplification [denaturation at 94°C for 30 sec, annealing at 54.5°C for 30 sec, extension at 68°C for 20 sec]. The resulting plasmid, termed E2-3A, was linearized with *Bam*HI and an antisense RNA probe generated by *in vitro* transcription in the presence of [α-^{32}P]UTP (NEN Life Science Products, Boston, MA) with the MAXIscript® T7 Kit (Applied Biosystems, Foster City, CA, USA). The antisense ^{32}P-riboprobe was 371 nucleotides (nt) in length, of which 319 nt were complementary to *Plp1* mRNA sequences. Thus, hybridization of the riboprobe to *Plp/Dm20* transcripts, and subsequent digestion with RNase A and RNase T1 would result in the protection of 319 nt. In addition, a 304 nt full-length antisense riboprobe for endogenous mouse β-actin sequences was produced with the pTRI-β-actin-Mouse template (Applied Biosystems), of which, 245 nt can be protected.

Full-length riboprobes were excised from 6% polyacrylamide/8 M urea gels and eluted in 350 μL of Elution Buffer (5 M ammonium acetate, 1 mM EDTA, 0.1% SDS) at 37°C for 3 h. RPAs were performed with the RPA III Kit (Applied Biosystems) according to the manufacturer's recommendations. In brief, 25 μg of total RNA from brain of mice at P9 to P390 of age, or 40 μg from younger animals (P2 through P7), was hybridized to ~8×10^5 cpm of ^{32}P-labeled riboprobes, overnight at 43.5°C. Afterwards, the reactions were treated with a mixture of RNase A/T1 and protected fragments fractionated on a 6% polyacrylamide/8 M urea gel. Dried gels were initially analyzed on a 445 SI PhosphorImager with ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA) and then subjected to autoradiography. Results are presented as the mean ratio of *Plp/Dm20* combined to β-actin (signal strength), with a minimum of 3 animals, per timepoint, per genotype.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Assessment of the relative levels of *Plp/Dm20* mRNA (combined) was also determined by real-time qRT-PCR analysis using the TaqMan Gene Expression Assay (Applied Biosystems). Single-stranded cDNAs were synthesized from total RNA (1.0 μg) using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) in a final reaction volume of 20 μL per manufacturer's specifications. Conditions for RT consisted of three (single) incubation steps: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. cDNAs were utilized immediately or stored at −70°C until needed. Quantitative PCR (qPCR) was carried out in a final reaction volume of 25 μL in iQ Supermix (Bio-Rad) containing 1 μL

(demyelination/remyelination study) or 2 μL (developmental study) of the RT reaction mixture (cDNAs), 900 nM of primers, and 250 nM of probe. Primer/probe sets were obtained from Applied Biosystems including one for β-actin (catalog No. 4352933E), which served as the reference gene. A custom primer/probe set was designed for *Plp1* [sense: 5′-CAAGACCTCTGCCAGTATAGG-3′; antisense: 5′-CAGCAATAAACAGGTGGAAGG-3′; probe: 6FAM-TGCCAGAATGTATGGTGTTCTCCCAT-MGBNFQ] which recognizes all splice variants (*Plp* and *Dm20*). qPCR was run (in triplicate) for 40 cycles (95°C for 15 s, 60°C for 1 min) in an iQ5 real-time PCR detection system (Bio-Rad) following a hot start (95°C for 10 min). Results for the developmental study were interpreted using the $2^{-\Delta\Delta C}$T method (Schmittgen and Livak 2008), and are reported as the fold difference relative to a uniform control (qRT-PCR product from pooled brain mRNA of P21 WT mice). mRNA levels with the cuprizone demyelination/remyelination study were calculated instead from a standard curve using the same uniform control (P21 WT mice), as previously described (Pereira *et al.* 2011). The ratio of *Plp1* (*Plp* and *Dm20* combined) to β-actin mRNA was calculated for each sample, for standardization. Results are presented as the percent value of corrected *Plp1* in cuprizone-treated mice relative to the average amount in untreated mice across all timepoints (within a genotype), which was arbitrarily set at 100%.

Western blot analysis

Preparation of whole brain lysates and Western blot analysis was performed as described previously (Li *et al.* 2009) with the following minor modifications. Proteins were denatured in Gel Loading Buffer (31.25mM Tris pH 6.8, 2% SDS, 5% glycerol, 0.05 mg/mL bromophenol blue, 0.785% β-mercaptoethanol) for 10 min at 55°C prior to fractionation on an SDS-PAGE gel (10% polyacrylamide), and subsequently transferred to a nitrocellulose membrane (Optitran BA-S85, Schleicher & Schuell, Florham Park, NJ, USA) per the manufacturer's recommendations. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST; 20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 1 h at 22°C, washed three times with TBST (15 min each), and subsequently incubated with primary mouse monoclonal antibodies. The anti-PLP/DM20 antibody (Abcam, Cambridge, MA, USA; catalog No. ab9311) was diluted 1 : 1000 in TBST plus 3% BSA and membranes were incubated overnight at 4°C. The anti-β-actin antibody (Abcam, catalog No. ab49900) was diluted 1 : 25,000 in TBST plus 5% non-fat dry milk and membranes were incubated for 1 h at 22°C. Blots were washed three times (5 min each) with TBST at 22°C and then incubated with secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) for 1 h. The anti-mouse secondary antibody was diluted 1 : 10000 in TBST plus 5% non-fat dry milk. Immunoreactive bands were visualized with the ECL Plus Western Blotting Detection System (GE Healthcare Biosciences, Piscataway, NJ, USA) and analyzed on a Storm 840 PhosphorImager with ImageQuant TL (GE Healthcare Biosciences).

Cuprizone-induced demyelination

Plp1$^{\Delta ASE}$ mice were crossed to C57BL/6J mice for seven generations prior to initiation of cuprizone-induced demyelination to enrich for the C57BL/6J genetic background. Animals were provided food and water available, *ad libitum*, throughout the study. Beginning at 8 weeks of age, male *Plp1*$^{\Delta ASE/Y}$ and *Plp1*$^{+/Y}$ littermates were fed 0.2% cuprizone [bis(cyclohexanone)oxaldihydrazone; Sigma-Aldrich Inc., St. Louis, MO, USA] in milled rodent chow for 6 weeks. Untreated (control) mice were fed milled chow (without cuprizone) during the same time period, after which both groups were returned to normal chow (pellets) for 1 to 3 weeks. Mice were anesthetized with isoflurane and brains quickly removed at 0, 1, 2, 3, 4, 5, 7, and 9 week's post-initiation of the study (cuprizone feeding). Brains were placed in a Petri dish (on ice) containing PBS and subsequently dissected

retaining the portion of tissue containing most of the corpus callosum – coronal sections at −0.22 and −0.82 mm from the bregma, followed by a horizontal cut at the midline just below the fornix, and retention of the dorsal piece. Total RNA was extracted from the tissue block and qRT-PCR analysis performed. Three animals per group (± cuprizone treatment/genotype) were investigated for every timepoint.

Statistical analysis

Statistical significance for differences in the levels of β-gal activity between transfected *Plp1-lacZ* constructs was determined by one-way ANOVA, with Tukey's post hoc analysis using the SAS 9.2 Software package (SAS, Cary, NC, USA). Bonferroni's post hoc analysis yielded similar interpretations (data not shown). Statistical significance for differences in *Plp1* gene expression between WT and ΔASE mice (RPA and qRT-PCR analyses) was determined by one-way ANOVA using the Generalized Linear Model (GLM) procedure from the SAS 9.2 Software package.

RESULTS

The ASE element is critical for high levels of *Plp1-lacZ* expression in oligodendroglial cell lines

As illustrated in Fig. 2a, the mouse *Plp1* gene contains seven major exons, with a relatively large first intron. A variety of *Plp1-lacZ* constructs have been generated to map regulatory elements within *Plp1* intron 1 DNA that are operative in N20.1 oligodendroglial cells. These constructs, depicted in Fig. 1, contain *Plp1* genomic DNA [2.4 kb 5′-flanking DNA, exon 1, and first 37 bp of exon 2] which is used to drive expression of a *lacZ* reporter gene cassette. The PLP(+)Z construct also includes all of *Plp1* intron 1 DNA, while PLPΔ809-5807 retains the intron except for positions 809-5807 based on numbering the intron from 1 to 8140 (Wight and Dobretsova 1997), and PLP(−)Z is missing the intron altogether. Similar to previous results (Dobretsova and Wight 1999), transfection analysis in N20.1 cells resulted in high levels of β-gal activity for the PLP(+)Z and PLP(−)Z constructs, and a much lower level for PLPΔ809-5807 [compare PLP(+)Z at 194% and PLPΔ809-5807 at 17% to PLP(−)Z, which was arbitrarily set at 100% for analysis in N20.1 cells]. Reinstatement of *Plp1* intron positions 1093-1177 at the deletion-junction site of PLPΔ809-5807 was able to rescue β-activity back to level with PLP(+)Z [181% for PLPΔ809-5807 + ASE], suggesting that the ASE is a potent positive regulatory element. PLPΔASE was constructed to test whether the ASE is the sole positive regulatory element within the intron.

The PLPΔASE construct is identical to PLP(+)Z except it is missing *Plp1* intron 1 positions 1084-1176 and contains a single *lox*P site in lieu of the ASE sequence. The *lox*P site was added to mimic the targeted *Plp1* gene rearrangement in mice as discussed later. Deletion of just the ASE resulted in low levels of β-gal activity in N20.1 cells (21% for PLPΔASE), which were similar to that with PLPΔ809-5807. Thus, the ASE appears to be the sole positive regulatory element from *Plp1* intron 1 that is operative in N20.1 cells.

To ascertain whether these findings are restricted merely to N20.1 cells, transfection analysis was also performed in Oli-neu cells. The Oli-neu cell line was derived by immortalizing enriched cultures of primary oligodendrocytes from mouse with the t-*neu* oncogene (Jung *et al.* 1995), and appears to be at a slightly later developmental stage than the N20.1 cell line (Pereira *et al.* 2011). Since the range of β-gal activity was so great between some of the constructs, transfection results with Oli-neu cells are reported as fold difference relative to PLP(−)Z, which was arbitrarily set at 1. As shown in Fig. 1, the level of β-gal activity with PLP(+)Z was 23-fold higher than that for PLP(−)Z in Oli-neu cells. Deletion of *Plp1* intron 1 positions 809 to 5807 resulted in low levels of β-gal activity (0.9-

fold for PLPΔ809-5807), similar to that for PLP(−)Z, suggesting that a positive regulatory element(s) lies within this 5 kb segment. Insertion of intron 1 positions 1093-1177 into the deletion-junction site of PLPΔ809-5807 was able to fully restore β-gal activity (22-fold for PLPΔ809-5807 + ASE) back to the level attained with PLP(+)Z, indicating that the ASE element is also operative in Oli-neu cells. Deletion of the ASE sequence alone resulted in moderately low levels of β-gal activity. Because the level of β-gal activity with PLPΔASE (5.1-fold) was still higher than that for PLPΔ809-5907 (0.9-fold), an additional positive regulatory element is active in Oli-neu cells between intron 1 positions 809-5807. Taken together, the transfection analysis in N20.1 and Oli-neu cells suggests that the ASE is a potent regulatory element through which an increase in *Plp1* gene activity can be mediated – at least in oligodendrocytes.

Generation of mice missing the ASE element from the *Plp1* gene (*Plp1*$^{\Delta ASE}$)

To understand the role that the ASE might play in modulating *Plp1* gene expression *in vivo*, intron 1 positions 1084-1176 were excised from the native gene in mouse using a Cre-mediated knock-in strategy (Fig. 2). Initially, a floxed Neo gene cassette was inserted in lieu of the ASE sequence via homologous recombination in ES cells with the targeting vector. Of the 201 ES clones screened, 5 clones demonstrated correct targeting (2.5% rate for homologous recombination). Four male chimeras (F_0 founders) were generated through injection of ES901 or ES914 ES clones into mouse blastocysts. Three of the chimeras [ES901/1 (80% agouti), ES901/2 (100% agouti) and ES914/2 (15% agouti)] sired pups with the agouti coat color indicating the possibility of germline transmission of the mutant *Plp*$^{Neo\Delta ASE}$ allele. Agouti progeny from the resulting F_1 generation were screened for presence of the mutant allele, which segregated in a Mendelian fashion. All F_1 agouti females were heterozygous for the mutant allele (*Plp*$^{Neo\Delta ASE/+}$) while F_1 agouti males were hemizygous for the WT gene (*Plp*$^{+/Y}$) as expected since male founders can only transmit the rearranged gene to female progeny; the *Plp1* gene is located on the X chromosome. Subsequently, F_1 females heterozygous for the *Plp1*$^{Neo\Delta ASE}$ allele were bred to EIIa-*Cre* transgenic mice to remove the Neo gene cassette (see Fig. 2c). Progeny positive for the *Plp1*$^{\Delta ASE}$ allele, which is identical to the native gene except it contains a single *lox*P site in lieu of the ASE (lacks 93 bp of intron 1), were backcrossed with the Cre-deleter for several more generations to ensure that the Neo cassette had been removed from all cells in the animal (data not shown).

The ASE is essentially dispensable for correct developmental regulation of *Plp1* gene expression in brain

To discern the role for the ASE *in vivo*, in context of the native gene, RPA analysis was performed with RNA isolated from male mice harboring the rearranged (*Plp1*$^{\Delta ASE/Y}$) or the WT (*Plp1*$^{+/Y}$) gene. Total RNA was obtained from the brains of mice at various ages (P2-P390) and the overall amount of *Plp/Dm20* transcripts (combined) relative to that for β-actin was determined for each animal (Fig. 3). No significant differences were noted except at P15, where the combined level of *Plp/Dm20* transcripts was 30% lower in ASE-deleted mice (*Plp1*$^{\Delta ASE/Y}$) compared to WT mice, however there was a tendency towards a reduction in the ASE-deleted mice during the first several weeks postnatal. Similar results were also obtained by qRT-PCR analysis, although no significant differences between the genotypes were noted (Fig. 4), which is not altogether surprising since small differences are generally lost (imperceptible) after PCR amplification. Likewise, Western blot analysis did not demonstrate any significant difference in the levels of PLP or DM20 in *Plp1*$^{\Delta ASE/Y}$ and *Plp1*$^{+/Y}$ mice at P15 to P35 of age (Fig. 5). These results suggest that the ASE is virtually dispensable for normal regulation of *Plp1* gene expression during development. Perhaps other elements in the *Plp1* gene, not present in the PLP(+)Z construct (Fig. 1), can functionally compensate for its loss.

Lack of the ASE does not significantly deter *Plp1* gene expression during remyelination

In demyelinating diseases, such as multiple sclerosis (MS), a combination of effects can lead to a failure in remyelination including depletion of myelinating cells, deficient recruitment of OPCs, and changes in gene expression programs (reviewed in Lubetzki *et al*. 2005). The myelin basic protein (*Mbp*) gene has been shown to contain multiple regulatory modules which are recruited selectively, in different combinations, to modulate *Mbp* gene activity during primary myelination, myelin maintenance, and remyelination in oligodendrocytes and Schwann cells (Farhadi *et al*. 2003). Since a single module (M4) was deemed to be important for regulating *Mbp* gene expression in oligodendrocytes during remyelination, we investigated if a similar scenario might be applicable with the ASE. Relative levels of *Plp1* gene activity in ASE-deleted and WT mice undergoing acute demyelination and subsequent remyelination were evaluated by qRT-PCR analysis (Fig. 6). To induce demyelination, 8 week old $Plp1^{\Delta ASE/Y}$ and $Plp1^{+/Y}$ mice were fed milled rodent chow containing 0.2% cuprizone for up to 6 weeks, and then switched back to normal rodent chow for up to 3 weeks. Similar to published results with this model (reviewed by Matsushima and Morell 2001), animals showed a marked decrease in *Plp/Dm20* mRNA levels following cuprizone ingestion (minimal at week 2), but after 5 weeks of treatment, the levels began to rise (Fig. 6). One week after discontinuation of cuprizone treatment (week 7 in Fig. 6), *Plp/Dm20* mRNA levels were back to pretreatment levels. While there was no significant difference in *Plp/Dm20* mRNA levels between ASE-deleted and WT mice, there was a hint that the levels in the ASE-deleted mice may have been somewhat restrained during the recovery (remyelination) phase (see weeks 5-9 in Fig. 6).

DISCUSSION

Mouse transgenesis with reporter constructs has greatly aided in the identification and characterization of *cis*-regulatory elements for a host of genes including CD69 (Vazquez *et al*. 2009), interleukin 4 (Yagi *et al*. 2007), *Mbp* (Foran and Peterson 1992), type X collagen (Zheng *et al*. 2009), and *Plp1* (Wight *et al*. 1993). However, as with any model, there are limitations. For instance, random integration of transgenes can lead to inadvertent position effects from the surrounding chromatin, thereby necessitating that multiple (independent) transgenic lines be scrutinized. Moreover, it is possible that additional regulatory elements, important for governing the expression of a particular gene, may not have been included in the transgene. To avoid these pitfalls, the ASE was targeted for deletion from the native (mouse) *Plp1* gene in order to study its role *in vivo*. While incorporation of the equivalent mutation (*lox*P site in lieu of the ASE) in a *Plp1-lacZ* construct (PLPΔASE) caused a dramatic decrease in expression in transfected N20.1 and Oli-neu cells (Fig. 1), loss of the ASE from the endogenous gene had, at most, a very minimal effect on *Plp1* gene activity during development; RPA analysis indicated that the combined level for *Plp/Dm20* transcripts in brain was 30% lower in ASE-deleted mice ($Plp1^{\Delta ASE/Y}$) compared to WT littermates ($Plp1^{+/Y}$) at P15 (Fig. 3). *Plp/Dm20* mRNA levels were similar at all other timepoints examined. Analysis by qRT-PCR did not reveal a difference in *Plp/Dm20* mRNA levels between the genotypes at any timepoint, although there was a hint that the levels in the ASE-deleted mice may have been somewhat tempered at P15 and P21 (Fig. 4). However, no significant difference was detected at the protein level (Fig. 5). Thus, the ASE is dispensable for appropriate *Plp1* gene regulation during development. This could be due simply to the fact that the ASE is not functional *in vivo*, or that other elements in the *Plp1* gene can functionally compensate for its lack. Accordingly, the ASE-deleted mice did not display any abnormal phenotype out to 16 months of age. Even the neurological/behavioral deficits observed in *Plp1* null mice are generally mild (for review see Rosenbluth *et al*. 2006).

The slight, but statistically significantly, decrease in *Plp/Dm20* mRNA levels for ASE-deleted mice at P15 by RPA analysis suggests that the ASE may actually be operational *in vivo.* It is possible that functionally redundant regulatory element(s) may not have been included in PLP(+)Z, which would explain why removal of the ASE from the *Plp1-lacZ* construct [PLPΔASE] led to such a dramatic reduction in its activity in the oligodendroglial cell lines (Fig. 1). The *MyoD* gene encodes an essential transcription factor responsible for muscle differentiation in vertebrates and contains functionally redundant *cis*-acting regulatory modules – a distal regulatory region (DRR) and a core enhancer – that can compensate for one another's loss (Chen *et al.* 2002; Chen and Goldhamer 2004). The *HoxD* gene (Zákány *et al.* 1997; Beckers and Duboule 1998) also exhibits functional redundancy in its transcriptional regulation. Thus, it is entirely likely that redundancy in gene regulation represents a safety mechanism, which ensures the expression of critical/important genes.

Alternatively, there could be another regulatory element present in *Plp1* intron 1 DNA that can compensate for loss of the ASE during most of development, but is not active at the stage consistent with N20.1 cells. Transfection analysis in Oli-neu cells, which appear to be at a later developmental stage than N20.1 cells by virtue of attaining a much higher level of PLP(+)Z expression relative to PLP(−)Z, seems to support this premise. Even though the ASE is a very potent positive regulatory element in Oli-neu cells, it is not the only positive regulatory element in *Plp1* intron 1 DNA; β-gal activity for PLPΔASE was still 5-fold higher than PLP(−)Z in Oli-neu cells (Fig. 1).

A couple of other regions in *Plp1* intron 1 having enhancer-like activity were identified using a controlled transgenesis approach that targeted reporter transgenes to the hypoxanthine phosphoribosyltransferase (*HPRT*) locus (Tuason *et al.* 2008). Specifically, highly conserved regions of mouse *Plp1* DNA were tested for their ability to support expression of a minimally promoted [basal heat shock protein (*hsp*) promoter] *eGFP-lacZ* reporter gene. A 1171 bp segment of intron 1 DNA (situated downstream of the ASE) led to transgene activity in mature oligodendrocytes in a temporal manner consistent with the endogenous *Plp1* gene, and as such, was named the wmN1 enhancer (Tuason *et al.* 2008). It is plausible that this element may be responsible for the slight elevation of β-gal activity in Oli-neu cells for PLPΔASE relative to PLP(−)Z, since removal of the just the wmN1 enhancer region from PLP(+)Z led to a modest decline in [PLPΔwmN1] activity (Fig. S1). Perhaps the wmN1 enhancer can counteract for loss of the ASE in the native gene at certain times of development, but is unable to fully do so in context of *Plp1-lacZ* constructs in transfected Oli-neu cells due to the lack of signaling from other cell types or the immature nature of the cell line itself. The wmN1 enhancer was found to be active during the latter stages of oligodendrocyte maturation (Tuason *et al.* 2008) and therefore may not be optimally active in Oli-neu cells. Additionally, another element was identified further downstream in *Plp1* intron 1 and named wmN2 (Tuason *et al.* 2008). The wmN2 enhancer was found to support relatively high levels of expression in the PNS when incorporated in the minimally promoted *eGFP-lacZ* transgene, while much lower levels were detected in white matter areas of the CNS. Interestingly, CNS expression of the transgene containing the wmN2 region was found to be maximal at P14, barely detectable at P30, and extinguished by P60 (Tuason *et al.* 2008), which happens to coincide with the timing of a maximal effect observed for *Plp1* gene regulation when the ASE is absent (Fig. 3). Perhaps the wmN2 enhancer can compensate for loss of the ASE in the native *Plp1* gene, but appears to be essential (much like the ASE) for attaining decent levels of *Plp1-lacZ* expression in Oli-neu cells (Fig. S2).

The ASE was also evaluated for enhancer activity in the controlled transgenesis study by Tuason *et al.* (2008). While the ASE was able to support expression of the *eGFP-lacZ* transgene in some (unidentified) cell types, it did not do so in oligodendrocytes,

oligodendrocyte progenitor cells (OPCs) or Schwann cells. Previously, we have shown that the ASE needs be in context of an intron in order to function in N20.1 cells. It lost its ability to augment expression of a reporter gene when repositioned upstream of the mouse *Plp1* promoter, or downstream of the (*lacZ*) reporter gene (Meng *et al*. 2005). Thus, it is not surprising that the ASE did not sustain expression in oligodendrocytes when placed upstream of the basal *hsp* promoter in the controlled transgenesis study by Tuason *et al*. (2008). Perhaps the ASE functions to enhance transcriptional elongation in oligodendroglial cells, which becomes immaterial when relocated to a site outside of the transcribed region.

It is conceivable that not enough sequence surrounding the ASE was removed to obliterate its activity in the ASE-deleted mice. However, in the study by Tuason *et al*. (2008), much larger fragments (2538 bp and 300 bp) of *Plp1* genomic DNA that encompass the ASE were tested by means of enhancer trap. Neither fragment [*ASE2538* and *ASE300*] directed transgene expression in oligodendrocytes, although transgene activity was detected in select regions of the CNS in other cell types. Hence, targeting a broader region of sequence for deletion of the ASE probably would not change the outcome of the present study, where loss of *Plp1* intron 1 positions 1084-1176 caused a significant reduction in expression of PLPΔASE in N20.1 and Oli-neu cells, but had little effect on expression of the native *Plp1* gene in mouse. While these seemingly contradictory results might be due a difference in chromatin structure between the two systems – transiently transfected cell lines and ASE-deleted mice – previously we have shown that the ASE is still able to augment expression of *Plp1-lacZ* constructs in N20.1 cells when stably transfected (Dobretsova *et al*. 2000). Thus, it appears that ASE activity is mediated via a factor(s) that acts as a true transcriptional activator rather than one that affects chromatin structure per se. Perhaps the disparate outcomes obtained here reflect a difference in transcription factor repertoires between the oligodendroglial cell lines and the ASE-deleted mice. Alternatively, loss of the ASE from the native *Plp1* gene may be compensated for by other functionally redundant elements in the gene which are either not present in PLPΔASE, or nonfunctional/not optimally active in N20.1 and Oli-neu cells. Maybe the oligodendroglial cell lines lack, or fail to properly activate, ASE-binding factor(s) due to the absence of an important signal such as those produced by neuron-glia interactions.

Similar to the situation during development, lack of the ASE from the native gene in mouse did not significantly alter *Plp1* gene expression after acute demyelination with cuprizone, although there was a hint that the levels may have been somewhat restrained in the ASE-deleted mice during the recovery (remyelination) phase (Fig. 6). Thus, the ASE is not the sole regulator of *Plp1* gene expression during remyelination. Whether this is due to compensation by another element (e.g., wmN1 enhancer) or whether the ASE is not important for *Plp1* gene expression during remyelination is unknown at this time.

In conclusion, the current studies demonstrate that the ASE is largely (if not entirely) dispensable for appropriate regulation of *Plp1* gene expression *in vivo*. This could be due to a difference between the transfection and transgenic paradigms. Namely, that the ASE is functional when examined by transfection analysis in cell culture, but this effect does not translate to the animal. On the other hand, transgenic studies (Li *et al*. 2002b) have clearly demonstrated the necessity of *Plp1* intron 1 DNA for accurate expression of *Plp1-lacZ* transgenes in mice. It is possible that another element in the intron (besides the ASE) may be wholly important or, alternatively, able to compensate for loss of the ASE from the native *Plp1* gene. As well, other elements, not present in the *Plp1-lacZ* transgenes, may have functional redundancy with the ASE. Because there was a modest decrease in *Plp/Dm20* mRNA levels with ASE-deleted mice at P15 (Fig. 3), and a hint of diminished levels at other times during development and during remyelination, the ASE may actually be active *in vivo*.

Whether this is due a weak effect imparted by the ASE itself or to compensation by another element in the *Plp1* gene remains to be seen.

Supplementary Material

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Abbreviations used

ASE	antisilencer/enhancer
β-gal	β-galactosidase
ES	embryonic stem;
floxed	flanked by *lox*P sites
HPRT	hypoxanthine phosphoribosyltransferase
HSV	herpes simplex virus
Mbp	myelin basic protein (gene)
MS	multiple sclerosis
n	number of samples
Neo	neomycin resistance
nt	nucleotides
OPC	oligodendrocyte progenitor cell
P	postnatal day
PGK	phosphoglycerate kinase
PMD	Pelizaeus-Merzbacher disease
PLP	myelin proteolipid protein
Plp1	myelin proteolipid protein (gene)
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RLU	relative light units
RPA	ribonuclease protection assay
SPG2	spastic paraplegia type 2
TBST	Tris-buffered saline with Tween-20
TK	thymidine kinase
WT	wild-type

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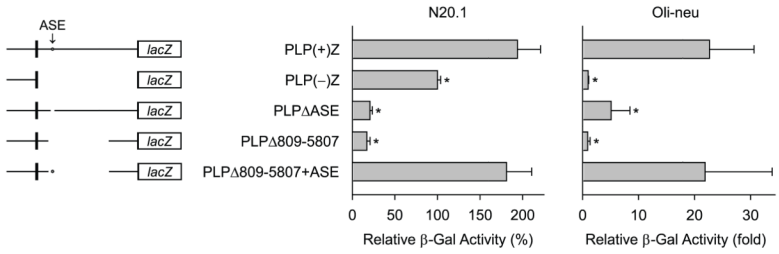

Fig. 1.

Transfection analysis of *Plp-lacZ* constructs in N20.1 and Oli-neu cells. Left: Schematic depiction of the *Plp1-lacZ* constructs used in this study (black boxes portray *Plp1* exon 1 and the first 37 bp of exon 2). The star portrays the ASE sequence in *Plp1* intron 1. Regions of *Plp1* intron 1 DNA missing from the *Plp1-lacZ* constructs are indicated by the absence of a line. PLP(+)Z contains the entire intron, whereas PLP(−)Z is missing the intron altogether. Right: Transfection results represent the mean ± SD of β-gal activity ($n \geq 6$) relative to PLP(−)Z, which was arbitrarily set at 100% (N20.1 cells) or 1-fold (Oli-neu cells) in every experiment. *Significant difference ($p < 0.05$) between PLP(+)Z and other constructs by one-way ANOVA with Tukey's post-hoc analysis.

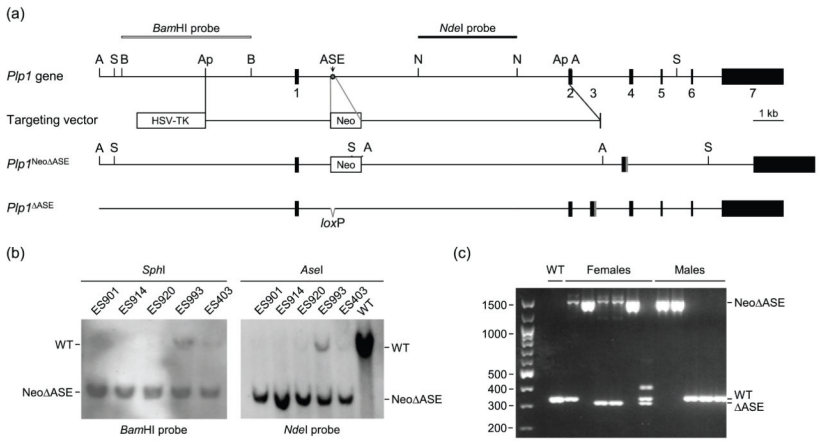

Fig. 2.

Strategy and validation for removal of the ASE from the native mouse *Plp1* gene. (a) Restriction map of the mouse *Plp1* gene is diagrammed at the top with only the pertinent restriction enzymes sites indicated: *Apa*I (Ap), *Ase*I (A), *Bam*HI (B), *Nhe*I (N), and *Sph*I (S). Numbered boxes depict the seven major exons. The terminal portion of exon 3 (gray box) encodes the PLP-specific region absent from DM20. The targeting vector contains sequences encoding selectable markers for negative (HSV-TK) and positive (Neo) selection to enrich for ES cells that underwent homologous recombination. The 'left arm' of the targeting vector contains 3.7 kb of *Plp1* DNA from the proximal *Apa*I site in the 5′-flanking region to intron 1 position 1083, while the 'right arm' encompasses sequence from intron 1 position 1177 to the *Apa*I site in exon 2. Thus, recombination with the targeting vector will result in deletion of the ASE (gray star) from the native *Plp1* gene, with a floxed Neo gene cassette in its place ($Plp1^{NeoΔASE}$). Incorporation of the Neo cassette introduces a new *Sph*I and *Ase*I site to the locus (only these site are indicated in the $Plp1^{NeoΔASE}$ allele). The Neo cassette was subsequently removed by crossing $Plp1^{NeoΔASE}$ mice with Cre-deleter mice (EIIa-*cre* C57BL/6 mice) to generate mice with the $Plp1^{ΔASE}$ allele, which contains a single *lox*P site in lieu of the ASE. (b**)** Validation of ES clones that underwent homologous recombination with the targeting vector by Southern blot analysis. Genomic DNA was isolated from the indicated ES clones and a wild-type (WT) control, digested with *Sph*I or *Ase*I, and hybridized to the *Bam*HI and *Nde*I probes, respectively. All of the ES clones shown contain the $Plp1^{NeoΔASE}$ allele (NeoΔASE). Clones ES920, ES993 and ES403 were also positive for the native *Plp1* allele (WT) to varying degrees, indicating contamination with ES cells that had not undergone homologous recombination (perhaps due to incomplete selection with G418 and/or ganciclovir). Clones ES901 and ES 914 were injected into 129/SvJ blastocysts and subsequently implanted in pseudopregnant C57BL/6 females. Resulting male chimeras (F_0 founders) were bred with C57BL/6 (WT) females and the F_1 progeny screened for the rearranged gene ($Plp1^{NeoΔASE}$). (c) PCR genotyping of progeny resulting from a parent harboring the $Plp1^{NeoΔASE}$ allele. Some of the female progeny contain the EIIa-*cre* transgene as well. Primers used for genotyping generate amplicons of 315 ($Plp1^{ΔASE}$), 333 (*Plp1*), and 1505 ($Plp1^{NeoΔASE}$) bp. Notice that removal of the Neo cassette was incomplete in two of the females; both ΔASE and NeoΔASE products were generated. The upper band (~400 bp) in the right lane of females is a hybrid between WT and ΔASE strands.

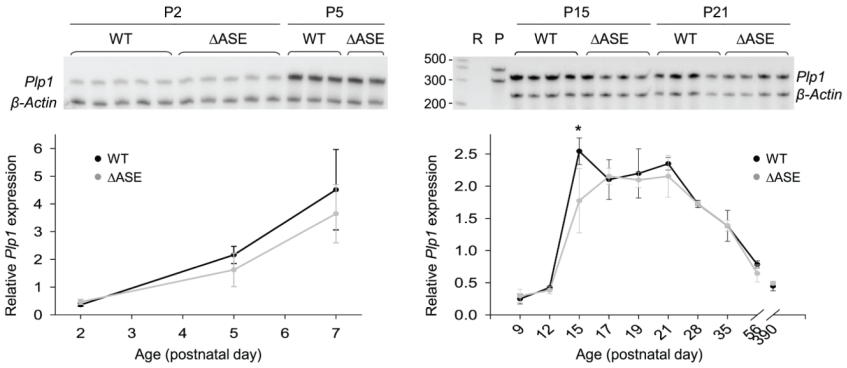

Fig. 3.

Developmental analysis (RPA) of *Plp1* gene expression in male WT ($Plp1^{+/Y}$) and ΔASE ($Plp1^{\Delta ASE/Y}$) mice. Total RNA was isolated from whole brain of mice at the indicated ages and hybridized to ^{32}P-labeled antisense riboprobes for *Plp1* (371 nt) and β-actin (304 nt). Forty micrograms of total RNA was used in the reactions with mice at P2-P7 of age, while only 25 μg was used for mice at P9-P390. Protected fragments of 319 nt (*Plp1*) and 245 nt (β-actin) result after digestion with RNase A and RNase T1. The *Plp1* riboprobe is unable to distinguish between *Plp* and *Dm20* mRNA species; therefore it is a measure of the combined value. The mean ratio ± SD of *Plp1* to β-actin mRNA levels is plotted for each timepoint/genotype as determined by PhosphorImager analysis ($n \geq 4$ for P2-P7; $n \geq 3$ for P9-P390). A representative autoradiogram is shown at the top of each plot. RPA analysis showed a significant decrease ($^{*}p < 0.0025$) in *Plp1* gene expression (*Plp* and *Dm20* mRNA levels combined) in ΔASE mice (30% lower) compared with WT mice at P15.

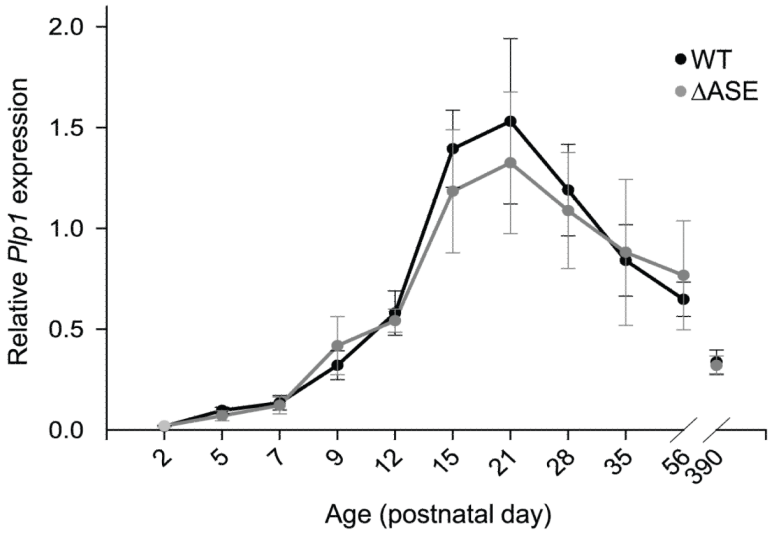

Fig. 4.

qRT-PCR analysis of *Plp1* gene expression in WT and ΔASE male mice from P2 to P390. A custom designed primer/probe set for *Plp1* was used, which detects all splice variants (*Plp* and *Dm20*). Results were obtained using the $2^{-\Delta\Delta C_T}$ method, with β-actin as the reference gene. Results are plotted as the mean level ± SD of *Plp1* (*Plp* and *Dm20* mRNA combined) relative to that from a uniform control (pooled brain mRNA from P21 WT mice) for each timepoint/genotype ($n \geq 4$ for P2-P7; $n \geq 3$ for P9-P390). While there tended to be a slight reduction in the level of *Plp1* gene expression in ASE-deleted (ΔASE) mice compared to WT littermates at P15 and P21, the difference was not significant.

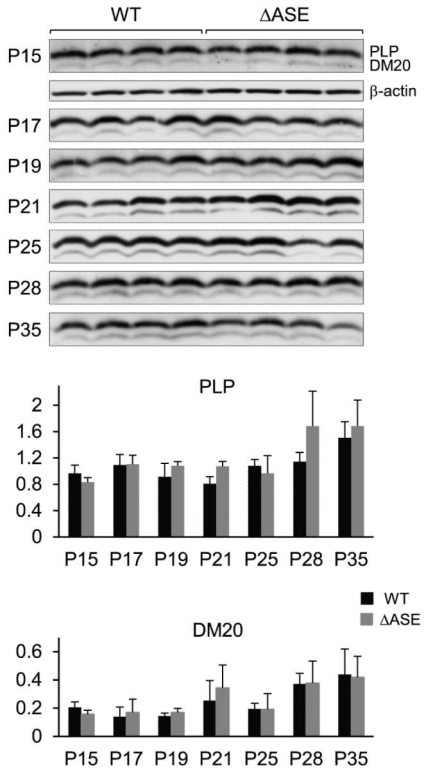

Fig. 5.

Western blot analysis of *Plp1* gene products in brain from WT and ΔASE male mice at P15-P35 of age. Protein was isolated from whole brain of mice at the indicated ages, and the relative levels of PLP and DM20 protein determined using an antibody that recognizes both isoforms. Four animals were evaluated for each timepoint/genotype. Blots were incubated first with an anti-PLP/DM20 antibody, and then stripped and incubated with an anti-β-actin antibody. Autoradiograms for PLP and DM20 are shown at the top for all timepoints; only the P15 timepoint is shown for β-actin due to space constraints. Plots represent the mean ratio ± SD of PLP or DM20 to β-actin ($n = 4$) for each timepoint/genotype by PhosphorImager analysis. No significant difference was noted between the levels of PLP or DM20 in WT and ΔASE mice.

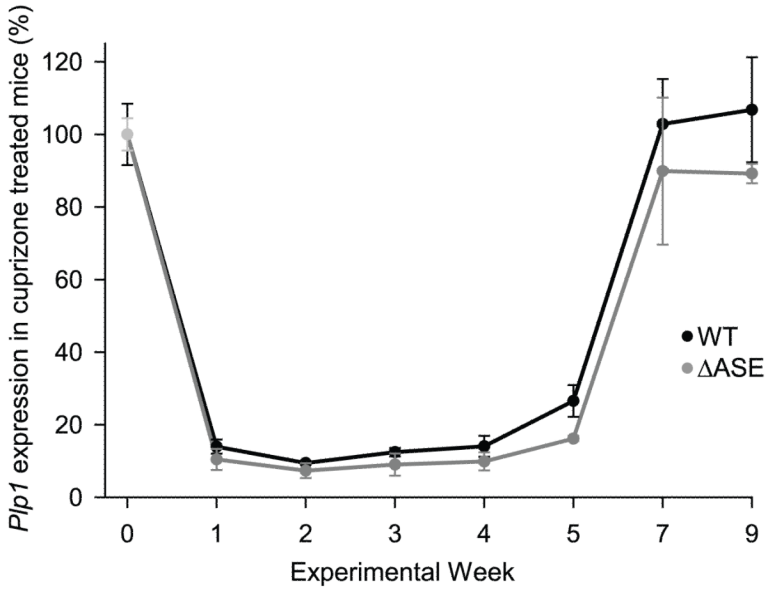

Fig. 6.

Cuprizone-induced changes in *Plp1* gene expression in WT and ΔASE mice. Mice at 8 weeks of age (week 0) were placed on a diet containing 0.2% cuprizone for up to 6 weeks (week 0-6) and then switched back to normal rodent chow for the remainder of the study. Untreated mice (not exposed to cuprizone) were also analyzed for both genotypes (WT and ΔASE). Animals were sacrificed at weekly intervals, and total RNA isolated from a defined block of tissue from the corpus callosum (see methods for more details). Real time qRT-PCR analysis was performed, and mRNA levels for *Plp1* (*Plp* and *Dm20* combined) and β-actin were determined from a standard curve generated with the same uniform control as in Fig. 4. The ratio of *Plp1* (*Plp* and *Dm20* combined) to β-actin mRNA was calculated for each sample, for standardization. Normalized values for *Plp1* in untreated mice (no cuprizone) were averaged across all timepoints and set at 100% for a given genotype (WT or ΔASE). Results are plotted as the mean value ± SD of corrected *Plp1* in cuprizone-treated mice relative to the average amount in untreated mice across all timepoints for a specific genotype ($n = 3$ for each timepoint/genotype).