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YY1 negatively regulates mouse myelin proteolipid protein (*Plp1*) gene expression in oligodendroglial cells

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ABSTRACT

YY1 (Yin and Yang 1) is a multifunctional, ubiquitously expressed, zinc finger protein that can act as a transcriptional activator, repressor, or initiator element binding protein. Previous studies have shown that YY1 modulates the activity of reporter genes driven by the myelin PLP (proteolipid protein) (*PLP1*/*Plp1*) promoter. However, it is known that *Plp1* intron 1 DNA contains regulatory elements that are required for the dramatic increase in gene activity, coincident with the active myelination period of CNS (central nervous system) development. The intron in mouse contains multiple prospective YY1 target sites including one within a positive regulatory module called the ASE (anti-silencer/enhancer) element. Results presented here demonstrate that YY1 has a negative effect on the activity of a *Plp1-lacZ* fusion gene [PLP(+)Z] in an immature oligodendroglial cell line (Oli-neu) that is mediated through sequences present in *Plp1* intron 1 DNA. Yet YY1 does not bind to its alleged site in the ASE (even though the protein is capable of recognizing a target site in the promoter), indicating that the down-regulation of PLP(+)Z activity by YY1 in Oli-neu cells does not occur through a direct interaction of YY1 with the ASE sequence. Previous studies with *Yy1* conditional knockout mice have demonstrated that YY1 is essential for the differentiation of oligodendrocyte progenitors. Nevertheless, the current study suggests that YY1 functions as a repressor (not an activator) of *Plp1* gene expression in immature oligodendrocytes. Perhaps YY1 functions to keep the levels of PLP in check in immature cells before vast quantities of the protein are needed in mature myelinating oligodendrocytes.

Key words: gene expression, gene regulation, myelin proteolipid protein gene, Oli-neu cells, repression, YY1.

INTRODUCTION

YY1 (Yin and Yang 1) is a multifunctional, ubiquitously expressed, zinc finger protein that can act as a transcriptional activator, repressor, or initiator element binding protein (for reviews see Shi et al., 1997; Thomas and Seto, 1999; Gordon et al., 2006; He and Casaccia-Bonnefil, 2008). YY1 is present in oligodendrocytes where it is localized to the nucleus (Rylski et al., 2008). The protein has been shown to recognize a target motif in the promoter of the human myelin PLP (proteolipid protein) gene (*PLP1*) where it seemingly functions as a transcriptional activator (Berndt et al., 2001). *PLP1*/*Plp1* gene expression is regulated in a spatiotemporal manner and, in oligodendrocytes, is responsible for nearly half the protein in CNS (central nervous system) myelin from adults (reviewed in Wight and Dobretsova, 2004). Categorization of YY1 as an activator of human *PLP1* gene transcription stems from studies by Berndt et al. showing that: (i) it binds selectively to a YY1 target site located within a region of the *PLP1* promoter referred to as site 3 [positions −130 to −104 relative to the transcription start point (+1)] (Berndt et al., 2001), which previously had been shown to bind nuclear protein(s) in a sequence-specific manner (Berndt et al., 1992); (ii) deletion–transfection analysis in glial cells using various *PLP1* promoter driven reporter constructs revealed a dramatic decrease in reporter gene activity when site 3 was deleted along with another, unrelated, protein binding site (site 2; positions −76 to −50) (Berndt et al., 1992); (iii) the level of expression of a *PLP1*-reporter gene construct that contains *PLP1* sequences from −1088 to +85 was increased when human (SVG) or rat (CG4) glial cell lines were co-transfected with a YY1 expression plasmid, but not with an analogous construct having a mutant site 3 that disrupts YY1 binding (Berndt et al., 2001). However, as noted by Berndt et al. (1992), the same (unmutated) *PLP1* sequence was not

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Abbreviations: ASE, anti-silencer/enhancer; β-gal, β-galactosidase; CMV, cytomegalovirus; CNS, central nervous system; DMEM/F-12, Dulbecco's modified Eagle medium/Ham's F-12 nutrient mixture; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; F-YY1, FLAG-tagged YY1; PLP, proteolipid protein; RLU, relative light units; RSVL, Rous sarcoma virus-luciferase; TBST, Tris-buffered saline with Tween-20; YY1, Yin and Yang 1.

effective in driving expression of a reporter gene in transgenic mice (Gout et al., 1991), whereas a related transgene that contains nearly 4.2 kb of 5′-flanking DNA could do so (Nadon et al., 1989, 1994). Discrepancies between the outcomes with transfection and transgenic approaches have been noted for other genes as well (Zimmerman et al., 1990; Kitsis and Leinwand, 1992; Donoviel et al., 1996), and may be due to differences in the chromatin status of the construct itself, or to inherent differences in the cells themselves.

The current study was undertaken to evaluate the role of YY1 in regulating mouse *Plp1* gene activity. The structure of the gene in mouse and man is quite similar (reviewed in Wight and Dobretsova, 2004). The 5′-flanking DNA is highly conserved between the two species, demonstrating 50% identity for the proximal 1.3 kb of sequence and 91% identity among the initial 135 nucleotides using the algorithm of Myers and Miller (1988). Moreover, the sequence orthologous to site 3 in the mouse *Plp1* gene [positions −132 to −106 based on the numbering system by Macklin et al. (1987)] is highly conserved, having 93% identity with the human sequence. Within this sequence there is an exact match to a high affinity CCAT core YY1 target site (consensus= VDCCATNWY) (Yant et al., 1995) that is identical to the corresponding sequence in man. However, unlike the case when the human *PLP1* promoter was used to drive a reporter gene in glial cell lines (Berndt et al., 1992), deletion of 'site 3' from the mouse *Plp1* promoter did not cause a major diminution in the levels of reporter gene activity (Wight et al., 1997), suggesting that this sequence does not function as a positive regulatory element in mouse. More recently it has been shown that overexpression of YY1 in the immortalized mouse oligodendroglial cell line, Oli-neu, causes a dose-dependent decrease in mouse *Plp1* promoter driven reporter gene activity when the cells were kept proliferating in growth medium (ODM) and 1% horse serum (He et al., 2007a). However, YY1 overexpression did not lead to a significant change in reporter gene activity when the cells were pressed to differentiate by dibutyryl cAMP treatment the day following transfection, except perhaps for a slight decrease with the highest amount (2 μg/well) of YY1 plasmid (He et al., 2007a). Thus, if anything, the mouse counterpart to site 3 (termed prom 3) appears to function as a negative regulatory element.

While the aforementioned studies have focused on effects of YY1 via regulatory sequences within the promoter and adjoining 5′-flanking DNA, the first intron of the mouse *Plp1* gene also contains many prospective YY1 binding sites (Wight and Dobretsova, 1997). Transgenic mouse studies (Li et al., 2002b) have shown that inclusion of *Plp1* intron 1 DNA in *Plp1-lacZ* fusion genes is critical to attain high levels of expression in brain during the active myelination period of CNS development, in keeping with the temporal pattern exhibited by the endogenous *Plp1* gene. Transfection analysis using a battery of *Plp1-lacZ* constructs containing partial deletion of *Plp1* intron 1 DNA revealed the presence of a single positive-regulatory element within the intron that is active in N20.1 cells (Dobretsova and Wight, 1999). Besides being able to overcome (or counterbalance) the effects from negative-regulatory elements located elsewhere in the intron, the positive-regulatory element functions as an enhancer in N20.1 cells (Dobretsova et al., 2000; Meng et al., 2005). Thus, we designated the positive regulatory element ASE (anti-silencer/enhancer). The ASE has been minimally mapped to mouse *Plp1* intron 1 positions 1093–1177 (Dobretsova et al., 2004) and contains an exact match to a high affinity YY1 target site (consensus=VDCCATNWY; Yant et al., 1995) spanning intron 1 positions 1137–1145. The intron also contains nine other potential YY1 binding sites with exact matches to the CCAT core consensus sequence. To address the effects that these sites might play in modulating *Plp1* gene expression, transfection analysis was performed in Oli-neu cells with *Plp1-lacZ* fusion genes containing all, some, or none of mouse *Plp1* intron 1 DNA. Oli-neu cells were used for this study because they are slightly more mature than N20.1 cells, based on the relative amounts and types of splice variants expressed by several myelin genes including *Plp1* (Pereira et al., 2011). In some experiments cells were co-transfected with an expression construct to increase the levels of YY1. Here we report that inclusion of *Plp1* intron 1 DNA greatly enhances the levels of *Plp1*-driven *lacZ* expression in Oli-neu cells. The increase in expression is largely attributable to the ASE regulatory element. However, overexpression of YY1 mitigates this response, apparently through an indirect mechanism. YY1 did not bind to the alleged target motif in the ASE as determined by gel shift analysis, although it did to a target site in prom 3 from the promoter. Both the ASE and prom 3 contain an exact match to the YY1 consensus sequence (VDCCATNWY). Therefore, the YY1 consensus sequence can be refined further based on differences in the sequence between ASE and prom 3.

MATERIALS AND METHODS

Cell culture

Dr Patrizia Casaccia (Mount Sinai School of Medicine) generously provided the mouse Oli-neu cell line (Jung et al., 1995) with permission from Dr. Jacqueline Trotter (University of Mainz). The Oli-neu cell line was derived by immortalization of primary cultures of enriched oligodendrocytes with the *t-neu* oncogene. Oli-neu cells were grown at 37°C in SATO medium devoid of mitogens (ODM) according to the modifications by He et al. (2007a) and supplemented with 1% horse serum. Oli-neu cells were maintained in an atmosphere of 10% CO_2. The mouse N20.1 cell line (Verity et al., 1993) was derived by immortalization of primary cultures of enriched oligodendrocytes with a temperature-sensitive form of SV40 large T antigen. N20.1 cells were grown at 34°C in DMEM/F-12 (Dulbecco's modified Eagle medium/Ham's F-12 nutrient mixture; Invitrogen) supplemented with 15 mM Hepes, 2.438 g/l of sodium bicarbonate, 4 g/l of glucose, 100

μg/ml of G-418 and 10% FBS (fetal bovine serum; HyClone), and maintained in an atmosphere of 5% CO_2. Monolayer cultures of HeLa cells were grown at 37°C in DMEM/F-12 supplemented with 15 mM Hepes, 2.5 mM L-glutamine, 5% FBS, 100 units/ml of penicillin G, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B, and maintained in an atmosphere of 5% CO_2.

Plasmids

Details regarding the construction of PLP(+)Z (Wight et al., 1993), PLP(−)Z (Wight and Dobretsova, 1997), and the *Plp1* intron 1 partial deletion constructs, PLPΔ12-8068 and PLPΔ809–5807 (Dobretsova and Wight, 1999), have been described previously. The constructs utilize the mouse *Plp1* promoter and associated sequences to drive expression of the *lacZ* reporter gene. Every *Plp1-lacZ* construct contains the proximal 2.4 kb of *Plp1* 5′-flanking DNA, all of exon 1 DNA, and the first 37 bp of exon 2. PLP(+)Z also contains all of *Plp1* intron 1 DNA, whereas PLP(−)Z lacks the intron altogether. The corresponding partial deletion constructs contain some of *Plp1* intron 1 DNA and were named according to the deleted intronic sequence. For instance, PLPΔ809–5807 is missing *Plp1* intron 1 DNA from positions 809–5807 based upon numbering the entire intron from positions 1–8140 (Wight and Dobretsova, 1997). Plasmid PLPΔ809–5807 +F(-AP4) (Dobretsova et al., 2004) contains the ASE sequence (*Plp1* intron 1 positions 1093–1177) inserted into the PstI site at the deletion–junction site of PLPΔ809–5807 in the native (forward; F) orientation, and is referred to here as PLPΔ809–5807+ASE-F for purposes of clarity.

F-YY1 (FLAG-tagged YY1) was expressed from the plasmid pCEP4F-YY1 (Yao et al., 2001), which contains a cDNA for human YY1 immediately downstream of the CMV (cytomegalovirus) promoter in pCEP4F (Zhu et al., 1995). The tagged protein contains a single copy of the FLAG epitope at its N-terminus.

Transfection analysis

Oli-neu cells were seeded at a density of 1×10^5 cells per 35-mm well (six-well dishes, Costar) the day prior to transfection. Cells were transfected with an equimolar amount of a given *Plp1-lacZ* construct and a fixed amount (0.35 μg/well) of a plasmid [RSVL (Rous sarcoma virus-luciferase)] containing the luciferase reporter gene under control of the LTR (long terminal repeat) promoter of RSV (rous sarcoma virus) to monitor for differences in transfection efficiency. Empty vector (pBluescript SK+; Stratagene) was added to bring the total amount of DNA to 3 μg per well. Each *Plp1-lacZ* construct was transfected in duplicate per experiment. In some cases, pCEP4F-YY1 was included as well. Cells were transfected using the FuGENE™ 6 Transfection Reagent (Roche Diagnostics Corp.) according to the manufacturer's specifications. Briefly, 4.5 μl of the FuGENE™ 6 Reagent was mixed with 95.5 μl of DMEM (without serum) before adding plasmid DNA (3 μg total in 16.7 μl of sterile water). The mixture was incubated for 1 h at room temperature and then added directly to the culture medium of an individual well. Cell lysates were prepared 48 h post-DNA addition in 190 μl of Reporter Lysis buffer (Promega). The Galacto-Light Plus Kit (Applied Biosystems) and Luciferase Assay System (Promega) were used to determine the relative levels for β-gal (β-galactosidase) and luciferase activity, respectively, as described earlier (Dobretsova and Wight, 1999). Luminescence was detected using an AutoLumat LB 953 luminometer (Berthold Technologies) as RLU (relative light units). Values for β-gal activity were corrected for differences in transfection efficiency first by subtracting the amount of background luminescence inherent in the β-gal and luciferase assays as determined by the RLU generated in lysates prepared from cells transfected with pBluescript SK+ alone, and then by adjusting the β-gal RLU in proportion to a set amount (2×10^6 RLU) of luciferase activity. Results are presented as the means ± S.D. of β-gal activity relative to that obtained for PLP(−)Z transfected cells, which was arbitrarily set at 1 (fold) in every experiment, and were calculated from at least three independent experiments. Alternatively, Oli-neu cells were transfected with pCEP4F-YY1 (0.1 μg/well) and pBluescript SK+ (2.9 μg/well) and nuclear extracts prepared 48 h post-DNA addition for subsequent use in EMSA (electrophoretic mobility shift assay) and Western-blot analysis. Statistical analyses were performed using the ANOVA module from SigmaPlot 11 (Systat Software Inc.).

Nuclear extracts

Nuclear extracts were prepared from cells (Oli-neu, N20.1, and HeLa) grown to ~80% confluence in 162 cm^2 flasks by the methods of Dignam et al. (1983). In some cases, nuclear extracts were prepared from 10 six-well plates of Oli-neu cells that had been transfected 48 h earlier with pCEP4F-YY1 (0.1 μg/well) and pBluescript SK+ (2.9 μg/well).

EMSA

EMSA analysis was performed as previously described (Dobretsova and Wight, 1999). EMSA probes were generated from synthetic double-stranded oligonucleotides (27-mers) that were radiolabelled using [γ-^{32}P]ATP (PerkinElmer) and T4 polynucleotide kinase (New England BioLabs). The sequence for one strand of each oligonucleotide is shown in Table 1 relative to a prospective YY1 binding site (consensus=VDCCATNWY). YY1 consensus and mutant oligonucleotides were obtained from Santa Cruz Biotechnology. Mouse *Plp1*-specific oligonucleotides were obtained from Integrated DNA Technologies. Prom 3 is orthologous to the human site 3 oligonucleotide used in an earlier study (Berndt et al., 2001) and spans the mouse *Plp1* promoter from positions −132 to −106 based on the numbering system utilized by Macklin et al. (1987). Prom 1 corresponds to *Plp1* 5′-flanking DNA positions −1596 to −1570 and contains two mismatches compared with the VDCCATNWY consensus, while still maintaining the CCAT core.

Table 1 **Oligonucleotides used in EMSA**

DNA sequences are shown only for the coding strand except for prom 3 which represents the non-coding strand. The YY1 binding site consensus sequence is VDCCATNWY (D=A or G or T; N=A, or C or G or T; V=A or C or G; W=A or T; Y= C or T). The nucleotides with matches to the YY1 consensus sequence are indicated in bold.

Region	Sequence
YY1 consensus	5′-CGCTCCCC**GGCCATCTT**GGCGGCTGGT-3′
YY1 mutant (mut YY1)	5′-CGCTCCGC**G**ATT**ATCTT**GGCGGCTGCT-3′
prom 1	5′-GTTCTTTTT**GCCATC**G**T**CCCTCTCCTC-3′
prom 3 (mouse site 3)	5′-CTTTAAGGG**CTCCATCTT**CTCTTTATG-3′
1128–1154	5′-ACCATGAAT**CACCATTTC**ATCATCTGG-3′

The 1128–1154 double-stranded oligonucleotide corresponds to *Plp1* intron 1 DNA position 1128–1154 (Wight and Dobretsova, 1997). Binding reactions for EMSA were assembled at room temperature in a total volume of 20 μl and consisted of 1–4 μg protein [nuclear extract alone or 0.1 μg 68 kDa YY1 polyhistidine-tagged fusion protein (Santa Cruz Biotechnology) plus 3.9 μg BSA], 1–2 μg poly(dI-dC)•(dI-dC) and $2–4 \times 10^4$ c.p.m. of labelled probe in a solution of 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 0.5 mM EDTA, 5% glycerol and 1 mM DTT (dithiothreitol). Some reactions also contained unlabelled 'competitor' DNA at 50-, 100- or 200-fold molar excess over the probe. DNA–protein complexes were resolved on non-denaturing 5% polyacrylamide gels and visualized by autoradiography.

Western-blot analysis

Proteins (10 μg from nuclear extracts) were denatured by heat (95°C for 5 min) in 50 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% Bromophenol Blue and 100 mM DTT (gel loading buffer). Proteins were fractionated on an SDS/PAGE gel (7.5% or 10% polyacrylamide) and subsequently transferred to a nitrocellulose membrane (Optitran BA-S 85, Schleicher & Schuell) using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) for 2 h at 100 V. Proteins were visualized by staining with Ponceau Red [0.1% (w/v) of Ponceau S (Sigma–Aldrich) in 5% acetic acid]. Membranes were subsequently destained and then blocked with 10% (w/v) non-fat dried skimmed milk powder in TBST (Tris-buffered saline with Tween-20; 20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) for 30 min at room temperature and washed three times (5 min each) in TBST. Membranes were incubated for 1 h with mouse monoclonal anti-YY1 (H-10) antibody (1:200 dilution; Santa Cruz Biotechnology) or anti-FLAG M2 antibody (10 μg/ml; Sigma–Aldrich) in TBST containing 3% BSA. Membranes were washed three times (5 min each) in TBST followed by incubation with secondary antibodies [HRP (horseradish peroxidase)-conjugated donkey anti-mouse IgG; Jackson ImmunoResearch Laboratories] diluted 1:5000 or 1:10000 in TBST with 5% non-fat dried skimmed milk powder. Membranes were washed three times (10 min each) in TBST and immunoreactive bands visualized using a chemiluminescence reagent kit (PerkinElmer).

RESULTS

The presence of *Plp1* intron 1 DNA greatly increases the level of *Plp1-lacZ* expression in Oli-neu cells, which is mitigated by elevated levels of YY1

Previous studies with *Plp1-lacZ* transgenic mice (Wight et al., 1993; Li et al., 2002b) have shown that inclusion of *Plp1* intron 1 DNA in the transgene is crucial to attain a robust developmental increase in β-gal activity in brain, concurrent with the active myelination period of CNS development. In particular, the PLP(+)Z transgene depicted in Figure 1, which utilizes *Plp1* genomic DNA [proximal 2.4 kb of 5′-flanking DNA downstream to the first 37 bp of exon 2] to drive *lacZ* reporter gene expression, is temporally regulated in brain similar to that of the endogenous *Plp1* gene. However, the developmental increase that is normally reached during the active myelination period is severely attenuated when *Plp1* intron 1 DNA is omitted from the transgene [PLP(−)Z construct depicted in Figure 1].

Deletion-transfection analysis with constructs equivalent to PLP(+)Z aside from missing a portion of *Plp1* intron 1 has identified a single positive regulatory element (ASE) and multiple negative regulatory elements that are functional in N20.1 cells (Dobretsova and Wight, 1999; Li et al., 2002a; Dobretsova et al., 2004). The cumulative effects of these regulatory elements are counterbalanced in N20.1 cells, hence PLP(+)Z and PLP(−)Z yield relatively similar levels of β-gal activity in transfected cells. However, as shown in Figure 2, the relative levels of β-gal activity in transfected Oli-neu cells is much greater for PLP(+)Z (∼50-fold higher) than PLP(−)Z. To test the effects that increased levels of YY1 might have on this ratio, Oli-neu cells were co-transfected with a YY1 expression plasmid (pCEP4F-YY1). Elevated levels of YY1, via co-transfection with only 0.025 μg pCEP4F-YY1, caused a dramatic drop in the β-gal activity for PLP(+)Z, which was not significantly different from that obtained with PLP(−)Z (Figure 2). Increased amounts of YY1 plasmid led to a further decrease, in a dose-dependent manner. Because PLP(+)Z and PLP(−)Z are the same except for the presence or absence of *Plp1* intron 1 DNA respectively the drop in β-gal

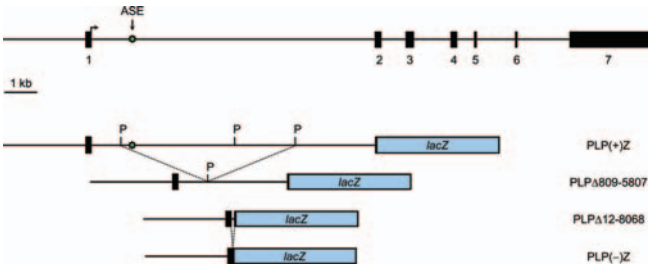

Figure 1 **Schematic diagram of the mouse *Plp1* gene and related *Plp1–lacZ* constructs**
The mouse *Plp1* gene is shown at the top and contains seven major exons (black boxes) that span 15.6 kb of DNA. There are also a couple of minor alternatively spliced exons (located in intron 1) that are not illustrated. The transcription start point is indicated by a bent arrow near the end of exon 1. A positive regulatory element called the ASE (green star) is located within the first intron at positions 1093–1177 (Dobretsova et al., 2004), based upon numbering the entire intron from positions 1–8140 (Wight and Dobretsova, 1997). *Plp1-lacZ* constructs pertinent to this study are depicted directly below the gene. All constructs contain 2.4 kb of mouse *Plp1* 5′-flanking DNA, exon 1 and the first 37 bp of exon 2, which is fused (in-frame) to a *lacZ* expression cassette (blue box). In addition, PLP(+)Z also contains all of *Plp1* intron 1 DNA, whereas PLP(−)Z is missing the intron altogether. The intron 1 partial deletion constructs contain a portion of *Plp1* intron 1 DNA and were named accordingly. For instance, PLPΔ809–5807 is missing intron 1 positions 809–5807, which was generated by the removal of two PstI (P) fragments from PLP(+)Z. PLPΔ809–807+ASE-F (not separately illustrated) contains the ASE sequence (positions 1093–1177) inserted at the deletion junction site (P) of PLPΔ809–5807 in the native (forward; F) orientation. Besides PLPΔ809–807+ASE-F, the only other construct which contains the ASE is PLP(+)Z.

activity for PLP(+)Z in the midst of elevated YY1 levels must be mediated through regulatory element(s) in the intron.

YY1 overexpression impairs ASE function

As noted earlier, the intron contains multiple potential YY1 binding sites, one of which is situated within the ASE. It is possible that overexpression of YY1 may interfere with the binding of a requisite activator whose recognition site overlaps or abuts the putative YY1 target site in the ASE.

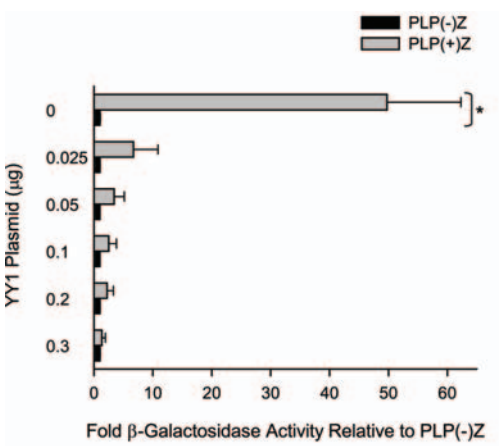

Figure 2 Overexpression of YY1 in Oli-neu cells causes a decrease in PLP(+)Z gene activity relative to that for PLP(−)Z in a dose-dependent manner
Oli-neu cells were transfected with equimolar amounts of a particular *Plp1-lacZ* construct in the presence or absence of the YY1 expression plasmid, pCEP4F-YY1 (Yao et al., 2001), along with a fixed amount of RSVL to correct for differences in transfection efficiency. Transfection results represent the -fold change in the means ± S.D. of β-gal activity ($n \geqslant 6$) for PLP(+)Z relative to PLP(−)Z, which was arbitrarily set at 1 in every experiment for each amount of pCEP4F-YY1 tested. Comparisons are made only among similar states of YY1 expression (i.e. same amount of transfected pCEP4F-YY1). *Significant difference ($P<0.001$) between PLP(+)Z and PLP(−)Z by one-way ANOVA with Bonferroni post hoc analysis.

To test this supposition, Oli-neu cells were co-transfected with equimolar amounts of a given *Plp1-lacZ* construct in the presence or absence of pCEP4F-YY1; 0.1 μg pCEP4F-YY1 was chosen since further decreases in PLP(+)Z gene activity with higher amounts of YY1 plasmid were minimal (Figure 2). β-gal activities are reported as the fold amount relative to that obtained with PLP(−)Z (arbitrarily set at 1) and compared only among the same backdrop of YY1 levels (normal or elevated). Similar to the results presented in Figure 2, overexpression of YY1 led to a significant decrease in the relative levels of β-gal activity in PLP(+)Z transfected cells compared with those transfected with PLP(−)Z (Figure 3). Removal of *Plp1* intron 1 sequence from positions 12–8068 [PLPΔ12–8068] or positions 809–5807 [PLPΔ809–5807] resulted in a dramatic decrease in the levels of β-gal activity compared with PLP(+)Z transfected cells containing endogenous levels of YY1 (i.e. not co-transfected with pCEP4F-YY1). Reinstatement of the ASE (positions 1093–1177) into the deletion–junction site of PLPΔ809–5807 [PLPΔ809–5807+ASE-F] was able to fully restore the levels of β-gal activity back to those obtained with PLP(+)Z (Figure 3). However, overexpression of YY1 caused a significant decrease in the levels of β-gal activity obtained with the PLPΔ809–5807+ASE-F construct (Figure 3). As shown in Figure 4, the amount of nuclear YY1 produced by the YY1 expression plasmid exceeded the endogenous level, and was discernible by an increase in molecular weight on account of the FLAG tag. Taken together, these data suggest that overexpression of YY1 has a negative effect on the action of the ASE in Oli-neu cells.

YY1 levels are similar in Oli-neu and N20.1 oligodendroglial cell lines

Because the ASE appears far more potent in Oli-neu cells compared with N20.1 cells [β-gal activity of PLPΔ809–5807+ASE-F transfected cells compared with PLP(−)Z was

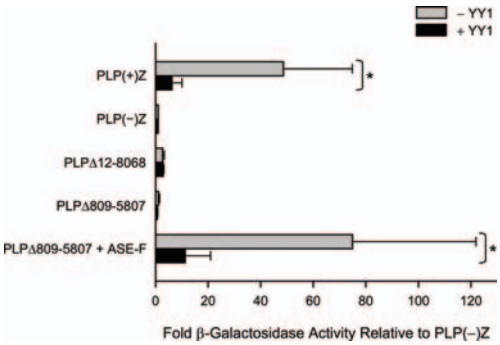

Figure 3 YY1 has a negative effect on ASE function in Oli-neu cells
Oli-neu cells were co-transfected with an equimolar amount of the indicated *Plp1-lacZ* construct (see Figure 1 for further details) and a fixed amount of RSVL to correct for differences in transfection efficiency. In some cases, 0.1 μg pCEP4F-YY1 was also included. Results represent the fold change in the means ± S.D. of β-gal activity (n=14) relative to that obtained with PLP(−)Z (arbitrarily set at 1 for each experiment), and compared only among equivalent states of YY1 expression. *Significant difference (P<0.001) between the expression of a given *Plp1-lacZ* construct in the presence and absence of co-transfected YY1 plasmid by one-way ANOVA with Bonferroni post hoc analysis.

approximately 75-fold higher in Oli-neu cells (Figure 3), while only 2-fold higher in N20.1 cells as previously shown (Dobretsova et al., 2004)] it is possible that the two cell lines have different amounts of YY1, which in turn leads to higher levels of *Plp1* gene expression in Oli-neu cells compared with N20.1 cells (Pereira et al., 2011). However, as shown in Figure 5, endogenous levels of YY1 in the nucleus are similar

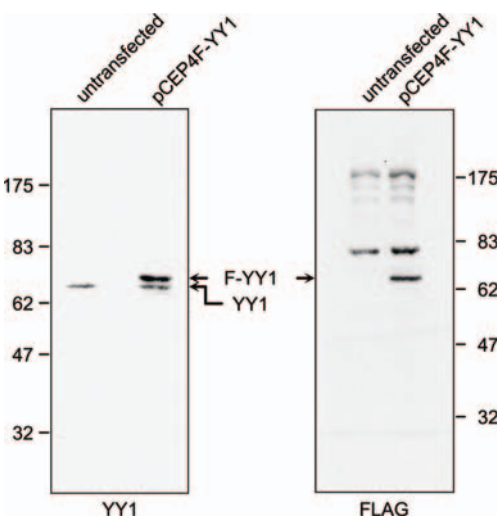

Figure 4 Transfection of Oli-neu cells with pCEP4F-YY1 results in relatively high levels of nuclear YY1, which exceed endogenous levels
Nuclear extracts were prepared from Oli-neu cells transfected with pCEP4F-YY1 (0.1 μg/well) 48 h earlier, or untransfected cells, and subsequently analysed for the presence of the YY1 protein (left panel) and the FLAG epitope (right panel) by Western-blot analysis. A total of 10 μg nuclear proteins were loaded per lane. The YY1 protein encoded by pCEP4F-YY1 contains a single copy of the FLAG epitope at its N-terminus (F-YY1), thereby increasing its molecular weight relative to the endogenous (YY1) protein.

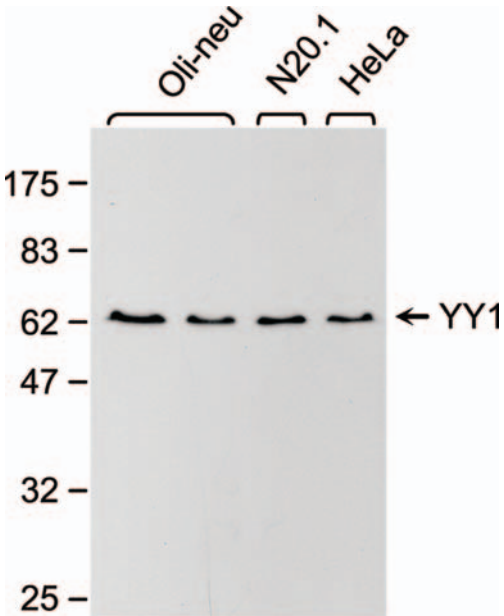

Figure 5 Oli-neu, N20.1 and HeLa cells contain comparable levels of nuclear YY1
Western blot analysis was performed using nuclear extracts prepared from Oli-neu, N20.1 and HeLa cells. Two independent isolations of nuclear extracts were tested for Oli-neu cells. An equal amount of nuclear proteins (10 μg) were loaded per lane; staining of the membrane with Ponceau Red indicated that the lanes contained comparable amounts of protein. Results show that the levels of nuclear YY1 are similar between the cell lines.

between Oli-neu and N20.1 cells, and even HeLa cells. Therefore the difference between the apparent potency of the ASE in Oli-neu and N20.1 cells cannot be explained by disparate levels of nuclear YY1.

YY1 binds to a motif in the mouse *Plp1* promoter, but not to the ASE

PCR assisted binding site selection has been used to define high-affinity YY1 binding motifs (Yant et al., 1995). The vast majority of high-affinity binding sites selected contained a core sequence of CCAT, although a few possessed a core sequence of ACAT. The consensus sequence, VDCCATNWY, was found to fit 89% of the selected CCAT-containing oligonucleotides (Yant et al., 1995). EMSA analysis was performed to test whether YY1 is capable of binding specifically to such recognition motifs present in the *Plp1* gene. As shown in Figure 6, a prominent DNA–protein complex was formed when nuclear proteins from Oli-neu cells were incubated with a positive control probe containing a single YY1 (consensus) binding site (lane 7). The complex was diminished by the addition of unlabelled homologous DNA (lanes 8–9), but not with a related mutant oligonucleotide (mut YY1) whose YY1 target site is disrupted (lanes 10–11). Furthermore, unlabelled prom 3 oligonucleotide (lanes 12–13), which corresponds to mouse *Plp1* promoter sequence from positions −132 to −106, could also compete for binding. Prom 3 is the mouse

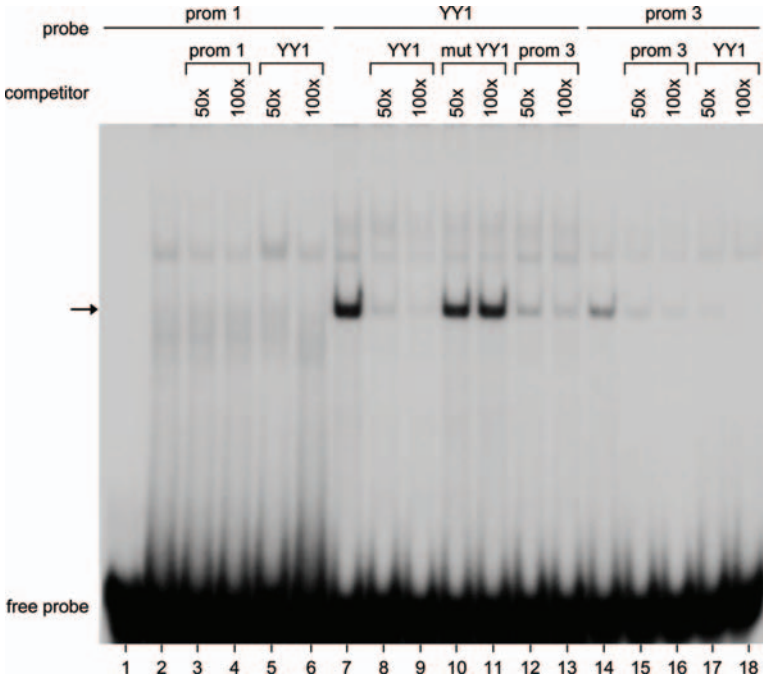

Figure 6 **YY1 may recognize a target site within the mouse *Plp1* promoter (prom 3)**
EMSA analysis was performed with the indicated ^{32}P-labelled probes and, in some instances, unlabelled DNA as a possible 'cold' (unlabelled) competitor (50- or 100-fold molar excess over the probe). See Table 1 for sequences of the oligonucleotides (27-mers) and their putative YY1 target sites. EMSA reactions with the prom 1 (lanes 1–6) and YY1 (lanes 7–13) probes contained a higher amount of radioactivity (4×10^4 c.p.m.) than those with the prom 3 probe (2×10^4 c.p.m.; lanes 14–18). Nuclear extract prepared from Oli-neu cells was included in all reactions (1.17 μg nuclear proteins/reaction; lanes 2–18) except for the sample in lane 1 (probe alone). Arrow points to a specific DNA–protein complex formed with the YY1 consensus and prom 3 probes.

equivalent of a human-based oligonucleotide that previously was shown to bind YY1 at site 3 in the *PLP1* promoter (Berndt et al., 2001), and fulfils the YY1 consensus. Likewise, when prom 3 was used as a probe, a similarly sized EMSA complex was formed (lane 14), which could be competed for by the addition of unlabelled YY1 consensus oligonucleotide, suggesting that YY1 can bind to this region of the mouse *Plp1* promoter. However, when an upstream oligonucleotide (prom 1) was tested that corresponds to mouse *Plp1* 5′-flanking DNA positions −1596 to −1570 and contains a CCAT core but two mismatches relative to the YY1 consensus, no DNA–protein complex was formed (lane 2). Hence, sequences flanking the CCAT core are also critical for YY1 binding.

EMSA analysis was also performed with nuclear extracts from Oli-neu cells that had been transfected with pCEP4F-YY1 two days earlier. As shown in Figure 7, there was enrichment of the EMSA sequence-specific complex formed by the YY1 consensus and prom 3 probes with nuclear extracts prepared from transfected cells (compare lanes 2 with 3, and 8 with 9), which were effectively diminished by addition of the YY1 consensus oligonucleotide as an unlabelled (cold) competitor (lanes 4 and 11). Incubation of the probes with 100 μg full-length human YY1 protein (produced in *Escherichia coli* as a 68 kDa polyhistidine tagged-fusion protein) led to an EMSA complex of nearly similar mobility whose level was greatly diminished by addition of unlabelled 'homologous' DNA (compare lanes 5 with 6, and 12 with 13). (The mobility of the EMSA complex formed with the purified YY1 protein was slightly retarded presumably due to the addition of the polyhistidine tag.) However, an EMSA complex was not formed between the purified YY1 protein and the 1128–1154 probe (lane 19) that contains a potential YY1 binding motif within the ASE sequence that fits the YY1 consensus exactly. Furthermore, even though some nuclear proteins from Oli-neu cells seem to recognize the 1128–1154 probe in a sequence-specific manner (compare lane 16 with lanes 17 and 18), it is not due to YY1 since the level of EMSA complexes remained unchanged when nuclear extracts from pCEP4F-YY1 transfected cells were tested (compare lanes 15 and 16), which was not diminished by the addition of unlabelled YY1 consensus oligonucleotide as a possible competitor (lane 18). Taken together, these results indicate that YY1 does not bind directly to the ASE sequence.

DISCUSSION

Past transfection studies (Berndt et al., 2001; He et al., 2007a) that tested the consequences of YY1 overexpression on the

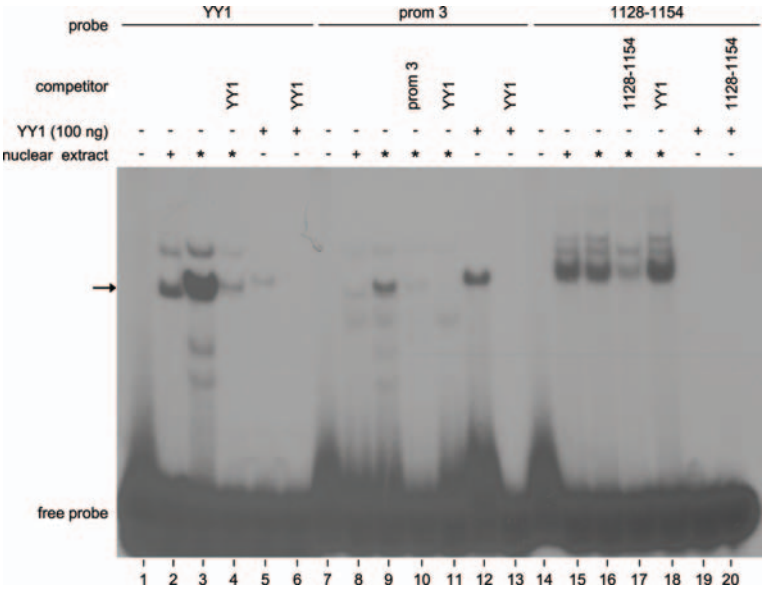

Figure 7 **YY1 does not bind directly to the ASE sequence, but does to a motif in the mouse *Plp1* promoter**
EMSA analysis was performed with the indicated ^{32}P-labelled probes (4×10^4 c.p.m./reaction). Unlabeled DNA (200-fold molar excess) was added to some reactions as a potential 'cold' competitor. See Table 1 for sequences of the oligonucleotides (double-stranded 27-mers). The respective probes were incubated either with nuclear extracts (4 μg nuclear proteins/reaction) prepared from untransfected (lanes 2, 8, 15) or pCEP4F-YY1 transfected (lanes 3–4, 9–11, 16–18) Oli-neu cells, or with purified polyhistidine tagged (human) YY1 protein (lanes 5–6, 12–13, 19–20), except for the samples in lanes 1, 7, and 13 (probe alone). Asterisks indicate nuclear extract prepared from transfected cells, which is the same as that used for the Western-blot analysis shown in Figure 4. Arrow points to a specific DNA–protein complex formed by nuclear proteins (most probably YY1) binding to the YY1 consensus and prom 3 probes. Reactions containing purified YY1 also formed a complex with slightly reduced mobility due to inclusion of a polyhistidine tag on the protein.

regulation of *PLP1/Plp1* gene expression have focused largely on effects mediated through sequences in the promoter and upstream 5′-flanking DNA. Yet YY1 has been shown to bind to target sites within the first intron of (other) genes and either activate (Kim et al., 2003, 2006, Foti and Reichardt, 2004) or repress (Yan et al., 2001; Zabel et al., 2002) their expression. Because mouse *Plp1* intron 1 DNA contains multiple putative YY1 binding sites (Wight and Dobretsova, 1997) and is essential for the generation of high levels of *Plp1-lacZ* transgene expression in brain during the active myelination period of CNS development (Li et al., 2002b), effects from elevated levels of YY1 on *Plp1-lacZ* gene activity via *Plp1* intron 1 sequences were investigated in the current study.

Previously it has been shown that targeted ablation of *Yy1* in oligodendrocyte progenitor cells through the use of *Cnp-cre* mice results in hypomyelination and cells being arrested at an immature stage (He et al., 2007a). The impediment in development is likely due to an inability to transcriptionally down-regulate the expression of oligodendrocyte differentiation inhibitors such as Id4, Tcf4 and Hes5 (He et al., 2007a; Shen et al., 2008); YY1 recruits HDAC1 (histone deacetylase 1) to the promoters of these inhibitors during oligodendrocyte progenitor differentiation causing 'repression of repressors', which in turn is required for differentiation to progress (He et al., 2007b). Concomitantly, there is an 80% reduction in mRNA levels for *Plp1*, MAG (myelin-associated glycoprotein), and UDP-galactose ceramide galactosyltransferase (*Ugt8*) in the brains of YY1 conditional knockout mice at postnatal day 18 (He et al., 2007a), presumably due to a failure in oligodendrocyte terminal differentiation.

On the other hand, YY1 overexpression in glial cells via transfection has produced a variety of effects, some of which are contradictory. Endogenous levels of *Plp1* gene transcripts in primary cultures of oligodendrocyte progenitors were largely unchanged when transfected with different amounts (0.5, 1 and 2 μg) of a plasmid (pCX-*yy1*) which expresses human YY1 (He et al., 2007a). However, it is unclear exactly what the transfection efficiency was in those experiments. If the efficiency was relatively low, then any changes in *Plp1* gene expression due to the elevation in YY1 levels would likely have been obscured by the high background of untransfected cells. Consequently, most studies have utilized *Plp1*-reporter gene constructs co-transfected with YY1 expression plasmids to determine how YY1 modulates *Plp1* gene activity. Ideally, another reporter plasmid such as RSVL would also be included in order to correct for differences in transfection efficiency between samples.

In the present study, where differences in transfection efficiency have been accounted for, overexpression of YY1 in Oli-neu cells caused a decrease in PLP(+)Z expression relative to that for PLP(−)Z (Figure 2). Given that both *Plp1-lacZ* constructs are equivalent except for the addition of *Plp1* intron 1 DNA in PLP(+)Z, the down-regulation of PLP(+)Z expression must have been mediated through regulatory

element(s) from the intron. Indeed, activity of the ASE (a very potent enhancer in Oli-neu cells) was thwarted by elevated levels of YY1 (compare the relative β-gal activities for PLPΔ809–5807+ASE-F in Oli-neu cells transfected with or without the YY1 expression plasmid in Figure 3). However, even though the ASE contains a potential YY1 binding motif that complies exactly with the VDCCATNWY consensus, YY1 did not bind to this sequence, although it could to another fit situated in the promoter (prom 3; Figure 6) analogous to the circumstances with site 3 in the human *PLP1* promoter (Berndt et al., 2001). [Analysis of the mouse *Plp1* sequence using MatInspector (Cartharius et al., 2005) revealed a YY1 motif in the sequence for prom 3, but not the ASE, using the consensus sequence CGCCATNTT as defined by Kim et al. (2007). Because both the prom 3 and 1128–1154 oligonucleotides contain a mismatch at position 2 compared with the CGCCATNTT consensus, but only the 1128–1154 oligonucleotide contains an additional mismatch (T→C) at the last position, it is likely that the terminal thymidine residue is important for YY1 binding.] Hence elevated levels of YY1 appear to counter ASE function (albeit not through direct binding to the ASE sequence itself), and consequently down-regulate *Plp1-lacZ* gene activity in Oli-neu cells. As well, YY1 overexpression in Oli-neu cells has been shown to have a negative effect on the activity of a reporter gene whose expression is driven wholly by the mouse *Plp1* promoter (and associated 5′-flanking DNA) (He et al., 2007a). Thus, expression of the mouse *Plp1* gene appears to be negatively affected by YY1. Curiously, the human promoter was positively regulated by YY1 in CG4 cells (rat oligodendroglial cell line), although increasing the amounts of YY1 plasmid transfected was inversely correlated with reporter gene activity (*PLP1*-CAT activity/μg protein) that had not been corrected for any deviations in transfection efficiency (Berndt et al., 2001). Whether these seemingly incongruent findings are the result of differences in the host cell (Oli-neu vs. CG4) and/or the relative amount of YY1 plasmid transfected to *Plp1*-reporter construct, or simply misleading because variations in transfection efficiency had not been accounted for in some studies, or truly due to a species-specific difference in the gene's regulation is presently unknown. Nonetheless, down-regulation of mouse *Plp1* driven reporter gene expression by YY1 is in line with the results obtained from mice with conditional ablation of *yy1* in oligodendrocyte lineage cells. He et al. (2007a) demonstrated that YY1 expression is necessary for oligodendrocyte progenitor differentiation. Presumably, YY1 is required to repress expression of terminal differentiation inhibitors. When oligodendrocyte progenitors isolated from the cortex of neonatal $Yy1^{floxed/floxed}$ mice were transduced with a CMV-Cre adenoviral vector and subsequently encouraged to differentiate by mitogen withdrawal, PLP immunoreactivity was much decreased in the YY1 ablated cells because they failed to differentiate. However, this does not mean, a priori, that YY1 is an activator of mouse *Plp1* gene expression. In fact, our results and those of He et al., (2007a) suggest that YY1 negatively influences its expression through sequences in *Plp1* intron 1 and the promoter, respectively. Yet, when Oli-neu cells are pressed to differentiate, elevated levels of YY1 did not significantly alter the activity of a mouse *Plp1* promoter-luciferase construct except for possibly a slight decrease with the highest amount (2 μg/well) of YY1 plasmid tested (He et al., 2007a). Thus the repressive effect of YY1 on *Plp1* gene expression appears to lessen (or be lost) as the cells move through the latter stages of differentiation. Perhaps YY1 helps to keep *Plp1* gene activity levels in check in immature cells before copious quantities of PLP are needed in myelinating oligodendrocytes.

Taken together, our results and those of others (He et al., 2007a) suggest that YY1 has a negative effect on mouse *Plp1* gene expression in oligodendrocyte progenitor cells and immature oligodendrocytes. The decrease in *Plp1* gene activity caused by YY1 is mediated in part through sequences present in the first intron, and with respect to the ASE does not occur via direct recognition of a YY1 target site. Perhaps elevated levels of YY1 cause a decrease in the expression of one or more of the activators that form a complex on the ASE. Alternatively, YY1 overexpression could diminish ASE activity by having an effect on the posttranslational modification of an ASE constituent, or bind via a protein–protein interaction and subsequently recruit a negative regulatory factor to the ASE complex. No matter what, this is above and beyond any effect mediated through the promoter (e.g. prom 3) since all of the *Plp1-lacZ* constructs used in the present study contain the same *Plp1* 5′-flanking DNA. It is worth mentioning that this effect was observed in the face of proportionately much less transfected YY1 plasmid compared with studies (Berndt et al., 2001; He et al., 2007a) that focused solely on the *PLP1/Plp1* promoter (and adjacent 5′-flanking DNA). In summary, YY1 appears to function as a negative regulator of mouse *Plp1* gene activity via multiple regions of the gene, including the first intron.

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