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Expression of Myelin Genes: A Comparative Analysis of Oli-neu and N20.1 Oligodendroglial Cell Lines

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Abstract

The use of immortalized cells has been instrumental as a tool in which to study gene regulation. However it is crucial to understand the status of a given cell line, especially when investigating the regulation of genes whose expression is developmentally regulated. Several immortalized cell lines have been derived from primary cultures of mouse oligodendrocytes. Two such cell lines – N20.1 and Oli-neu – were characterized here in terms of their relative expression of myelin genes at both the mRNA and protein level. Analysis of the splice isoforms expressed by the myelin proteolipid protein (*Plp1*), myelin basic protein (*Mbp*), and 2',3'-cyclic nucleotide 3'-phosphodiesterase (*Cnp*) genes, along with the relative amount of protein expressed by these genes suggest that the cell lines are representative of immature oligodendrocytes, although Oli-neu cells appear to be further along the differentiation pathway compared to N20.1 cells. Previous studies have shown that the developmental increase in *Plp1* gene expression that occurs during the active myelination period is governed by transcription regulatory elements present within the first intron. The responsiveness of one of these elements – the so-called antisilencer/enhancer (ASE) – was investigated in both cell lines. Results presented here suggest that the ASE has a much more potent effect in Oli-neu cells. Thus, the two cell lines appear to be at different stages and will be useful as a means to study transcription regulatory elements whose influence changes during development.

Keywords

myelin proteolipid protein; myelin basic protein; CNP; gene expression; oligodendrocyte; enhancer

INTRODUCTION

Oligodendrocytes are highly specialized cells that are responsible for the production of myelin in the central nervous system (CNS). During development, oligodendrocyte progenitor cells migrate from discrete regions in the developing brain and spinal cord to emergent areas of white matter. Upon interaction with neurons, oligodendrocytes can fully differentiate into a myelinating phenotype, capable of producing copious amounts of myelin which ensheathes segments of axons (internodes) thus allowing for saltatory conduction of nerve impulses. The protein composition of CNS myelin is relatively simple with gene products encoded by the myelin proteolipid protein (*Plp1*), myelin basic protein (*Mbp*), and 2',3'-cyclic nucleotide 3'-phosphodiesterase (*Cnp*) genes accounting for roughly 50%, 30%

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and 4% of total myelin proteins, respectively (reviewed by Baumann and Pham-Dinh, 2001), although a recent study (Jahn et al., 2009) suggests that the amounts of PLP and MBP may be somewhat less. Here we report the relative expression of these genes in two immortalized mouse oligodendrocytic cell lines.

While primary cell cultures have yielded much to our understanding of oligodendrocytes, they can be challenging to use for delineation of transcription regulatory elements since primary cells are difficult to transfect and do not propagate well, thus creating a relentless demand for fresh tissue. As a result, immortalized cells have been the cell of choice in which to map transcription regulatory elements. Immortalized oligodendroglial cell lines have been generated via two methods; most commonly via forced expression of an oncogene (Verity et al., 1993; Jung et al., 1995), and occasionally by continual passage of precursor cells in the presence of growth factors (Louis et al., 1992). Two cell lines, derived through immortalization of mouse primary oligodendroglial cells with oncogenes, were used in the current study. A temperature-sensitive form of SV40 T antigen was used to generate the N20.1 cell line, which allows for proliferation or differentiation at the permissive and nonpermissive temperatures, respectively (Verity et al., 1993). The *t-neu* oncogene, which differs from the *c-neu* proto-oncogene by a point mutation rendering the tyrosine kinase constitutively active, was used to generate the Oli-neu cell line (Jung et al., 1995). These cells have been shown to differentiate *in vitro* and *in vivo* and express antigen markers consistent with immature oligodendrocytes (Jung et al., 1995). Expression of a gene within a cell line indicates that the cells contain the requisite nuclear factors needed for its expression, thus confirming that the cell line is an appropriate model in which to study the regulation of that particular gene. In the current study, expression of *Plp1*, *Mbp* and *Cnp* genes were determined at the RNA and protein levels in N20.1 and Oli-neu cells as a means to estimate the relative developmental status between the cell lines. Previous studies with transgenic mice have demonstrated that the first intron of the *Plp1* gene contains regulatory elements responsible for governing its developmental expression in brain (Li et al., 2002). Deletion-transfection analysis in N20.1 cells identified a potent positive regulatory element located between *Plp1* intron 1 DNA positions 1093 and 1177 (Dobretsova et al., 2004). We named the element ASE, for antisilencer/enhancer, since it seemed to override repression mediated by negative regulatory elements that reside elsewhere in the intron (Dobretsova and Wight, 1999). In the current study, the relative influence the ASE has on *Plp1* gene expression in N20.1 and Oli-neu cells was investigated, as well as the outcome from the disruption of a potentially critical AP-1-like binding site within the ASE.

MATERIAL AND METHODS

Cell Culture

The immortalized mouse oligodendrocytic cell line, N20.1, was graciously provided to us by Dr. Anthony Campagnoni (University of California at Los Angeles). 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) 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) and maintained in an atmosphere of 5% CO₂. The immortalized mouse oligodendrocytic cell line, Oli-neu, was kindly provided by Dr. Patrizia Casaccia-Bonnet (Mount Sinai School of Medicine) with permission from Dr. Jacqueline Trotter (University of Mainz). Oli-neu cells were grown at 37°C in SATO medium containing 1% horse serum (Invitrogen), and maintained in an atmosphere of 10% CO₂.

Quantitative Reverse Transcription - Polymerase Chain Reaction (qRT-PCR) Analysis

N20.1 and Oli-neu cells were grown to approximately 80% confluence in a T-150 flask, washed twice with PBS, and total RNA extracted using the TRIzol Reagent (Invitrogen) according to the manufacturer's specifications. The concentration of RNA was calculated from the optical density obtained at 260 nm in a spectrophotometer. Subsequently, cDNA synthesis was performed with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) per the supplier's instructions using 1.0 µg of total RNA in a final reaction volume of 20 µl. The RT-PCR program began with a single step of 25°C for 5 min, followed by a single step of 42°C for 30 min, and a final step of 85°C for 5 min. cDNAs were utilized right away or stored at -70°C until needed. Gene expression analysis was performed using the TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA). A custom primer/probe set was designed for *Plp* and *Dm20* combined (forward primer 5'-CAAGACCTCTGCCAGTATAGG-3'; reverse primer 5'-CAGCAATAAACAGGTGGAAGG-3'; probe 6FAM-TGCCAGAATGTATGGTGTCTCCCAT-MGBNFQ). All other primer/probe sets were commercially available from Applied Biosystems (β -actin, catalog No. 4352933E; *Dm20*, catalog No. Mm01297211_m1; *Mbp*, catalog No. Mm01266402_m1 and Mm01262036_m1; *Cnp*, catalog No. Mm01306640_m1). Quantitative PCR (qPCR) was carried out in a final reaction volume of 25 µl containing IQ™ Supermix (Bio-Rad), 2 µl of the RT reaction mixture (single-stranded cDNA), 900 nM of primers, and 250 nM of probe. qPCR was run for 40 cycles (95°C for 15 sec, 60°C for 1 min) in an IQ™ 5 Real-Time PCR Detection System (Bio-Rad) following a hot start (95°C for 10 min). Each run consisted of (in triplicate) a 5 point standard curve (series of 5-fold dilutions), a 'no template' negative control, and cDNA from both cell lines, for all primers tested. The same source of pooled N20.1 cDNA was used to generate a standard curve for every experiment. The amount of mRNA produced by a specific gene in the two cell lines was determined from the standard curve with the IQ™5 software. The level of β -actin mRNA served as a reference point for every experiment. Results are presented as the mean amount (ng) of mRNA of the test gene, relative to that for β -actin from a given sample. Similar trends were also observed when the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) primer/probe set (Applied Biosystems, catalog no. Mm99999915_g1) was used as the reference gene instead of β -actin (data not shown).

Western Blot Analysis

N20.1 and Oli-neu cells were grown to approximately 80% confluence in a T-150 flask. Cells were washed twice with PBS and then scraped into a 2-ml solution of 4% SDS. Cells were ruptured by 20 strokes in an all-glass Dounce homogenizer, and lysates cleared by centrifugation at $16,000 \times g$ for 5 min. Protein concentrations for the resulting supernants were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL), and measured simultaneously to decrease variability between samples. A protein extract was also prepared as previously described (Li et al., 2009) from mouse (postnatal day 35, P35) brain for use as a reference. Western blot analysis was performed as described previously (Dobretsova et al., 2008) with the following minor modifications. Proteins in the lysate were denatured by heating at 55°C for 10 min in 31.25 mM Tris pH 6.8, 5% glycerol, 0.05 mg/ml bromophenol blue, 0.857% β -mercaptoethanol (Gel Loading Buffer without SDS) prior to loading on an SDS-PAGE gel (10% polyacrylamide). After electrophoresis, proteins were transferred to a nitrocellulose membrane by electro-transfer. Membranes were blocked with 5% non-fat dry milk in TBST (20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 1 hr at room temperature, washed three times (15 min each) in TBST, and subsequently incubated with primary antibodies. Primary monoclonal antibodies (Abcam Inc., Cambridge, MA) and incubation conditions were as follows: mouse anti-PLP/DM20 (catalog No. ab9311) or rat anti-MBP (catalog No. ab7349) antibody diluted 1:1000 in TBST containing 3% BSA,

incubated overnight at 4°C; mouse anti-CNP antibody (catalog No. ab6319) diluted 1:2000 in TBST containing 3% BSA, incubated for 1 hr at room temperature; mouse anti-β-Actin antibody (catalog No. ab49900) diluted 1:25,000 in TBST containing 5% non-fat dry milk, incubated for 1 hr at room temperature. Membranes were washed three times (5 min each) in TBST at room temperature and then incubated with secondary antibodies (anti-mouse or anti-rabbit antibody, Jackson ImmunoResearch, West Grove, PA; anti-rat antibody, Abcam Inc.) for 1 hr. Secondary antibodies were diluted in TBST containing 5% non-fat dry and used at the supplier's recommended dilution for Western blot application. Immunoreactive bands were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare Biosciences, Piscataway, NJ) and analyzed on a Storm 840 PhosphorImager with ImageQuant TL (GE Healthcare Biosciences).

***Plp1-lacZ* Constructs**

Generation of many of the *Plp1-lacZ* plasmids used in this study has been described previously. All plasmids are identical to PLP(+)*Z* except for deletion of some or all of *Plp1* intron 1 DNA. PLP(+)*Z* (Wight et al., 1993) contains mouse *Plp1* genomic DNA, extending from the proximal 2.4 kb of 5'-flanking DNA down to the first 37 bp of exon 2, which is used to drive expression of a *lacZ* reporter gene cassette. Plasmid PLP(-)*Z* (Wight and Dobretsova, 1997) is missing all of *Plp1* intron 1 DNA, whereas PLPΔ809-5807 (Dobretsova and Wight, 1999) is missing only intron 1 positions 809 to 5807 based on numbering the entire intron from 1 to 8140 (Wight and Dobretsova, 1997). Plasmids PLPΔ809-5807 + F(-AP4) and PLPΔ809-5807 + R(-AP4) contain 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) or reverse orientation, respectively (Dobretsova et al., 2004), and are referred to here as PLPΔ809-5807 + ASE-F and PLPΔ809-5807 + ASE-R for simplicity. Two similar constructs containing a disrupted AP1 site were generated by insertion of a double-stranded *Plp1* intron 1-specific (positions 1093-1177) oligonucleotide possessing a 3-bp mutation (AAT→CGA¹¹³⁴⁻¹¹³⁶) into the PstI deletion-junction site of PLPΔ809-5807, according to standard DNA recombination techniques (Sambrook and Russell, 2001). The insert was generated by annealing DNA oligonucleotides (Integrated DNA Technologies, Coralville, IA) with following sequences: sense strand, 5'-GTC TCT AAG GAA CTC TGC AAA ATT AGG CAT GTC ACC ATG **CGA** CAC CAT TTC ATC ATC TGG TAG ACA GGG GCA ACA ATA ACT GTA TGC A-3'; antisense strand, 5'-TAC AGT TAT TGT TGC CCC TGT CTA CCA GAT GAT GAA ATG GTG **TCG** CAT GGT GAC ATG CCT AAT TTT GCA GAG TTC CTT AGA GAC TGC A-3' (mutated sequence in bold, PstI staggered ends underlined). DNA sequencing was used to determine the orientation of the insert and to confirm that only a single copy of the insert had been added. The constructs were named PLPΔ809-5807 + mutASE-F and PLPΔ809-5807 + mutASE-R to signify the orientation of the mutated ASE sequence relative to the remainder of *Plp1* intron 1 DNA.

Transfection Analysis

N20.1 cells were transiently transfected as previously described (Dobretsova and Wight, 1999). Briefly, N20.1 cells were seeded at a density of 2.8×10^5 cells per 35-mm well (six-well plate; Costar, Cambridge, MA) 16–18 hr prior to transfection in 2 ml of growth medium. The cells within a given well were transfected with 2 μg of total DNA and 5 μl of Lipofectamine (Invitrogen) according to the manufacturer's specifications. Cells were independently transfected with equimolar amounts of *Plp1-lacZ* constructs or with the empty vector (pBluescript SK+; Stratagene, La Jolla, CA) alone. pBluescript SK+ was added as necessary to keep the total amount of DNA constant. A fixed quantity (0.35 μg/well) of RSVL (de Wet et. al., 1987) was also included to control for differences in transfection efficiency. N20.1 cells were incubated with the DNA/Lipofectamine mixture for

6 hr and then washed two times with growth media. Lysates were prepared approximately 72 hr post-DNA addition in 190 μ l of Reporter Lysis Buffer (Promega, Madison, WI) as recommended by the supplier.

Oli-neu cells were seeded at a density of 2.5×10^5 cells per 35-mm well, 16–18 hr prior to transfection in 2 ml of growth medium. The cells within a given well were transfected with 3 μ g of total plasmid DNA (equal to the amounts of *Plp1-lacZ* and RSVL constructs used in N20.1 cells, with increased quantities of pBluescript SK+) and 4.5 μ l of FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer's specifications. All constructs were transfected in duplicate (or multiples thereof) per experiment. Cells were incubated for 48 hr post-DNA addition (no washout), and lysates prepared thereafter as described earlier for N20.1 cells.

Aliquots of lysate (10 μ l each) were evaluated for reporter gene expression in triplicate using the Galacto-Light Plus Kit (Tropix, Bedford, MA) and Luciferase Assay System (Promega) for determination of β -gal and luciferase activities, respectively, as previously described (Dobretsova and Wight, 1999). Luminescence was measured as relative light units (RLU) with an AutoLumat LB 953 luminometer (EG&G Berthold, Gaithersburg, MD). The background levels of luminescence were determined with cells transfected solely with pBluescript SK+ and subtracted from all measurements, accordingly. Differences in transfection efficiency were corrected for by adjusting the level of β -gal activity relative to a set amount (2×10^6 RLU) of luciferase activity. Results are presented as the normalized mean $\pm$ SD of β -gal activity relative to that obtained for PLP(-)Z transfected cells, which arbitrarily was set at 100% for N20.1 cells, and 1-fold for Oli-neu cells, in every experiment. Transfection results represent three or more independent experiments. Data were analyzed by one-way ANOVA and post-hoc using SAS[®] 9.2 Software (SAS Institute Inc., Cary, NC).

RESULTS

Immortalization of primary oligodendroglial cells with oncogenes has resulted in the generation of important models of oligodendroglial lineage cells that can be utilized to investigate molecular and cellular features germane to these cells. Two such mouse-derived cell lines, N20.1 and Oli-neu, were used in the current study. The relative expression of major myelin genes was compared between the cell lines. Each line was maintained under select culture conditions throughout the study, which empirically were determined to be compatible with transient transfection procedures. The functional contribution that an intronic enhancer (ASE) has on *Plp1* gene activity was also evaluated in the two cell lines.

mRNA levels for myelin genes are higher in Oli-neu cells compared to N20.1 cells

Real-time RT-PCR (qRT-PCR) analysis was performed to compare the levels of mRNA for the *Plp1*, *Mbp*, and *Cnp* genes in N20.1 and Oli-neu cells. Values are reported as the amount relative to that for β -actin, although similar trends were also observed when *Gapdh* was used as the reference housekeeping gene (data not shown). The *Plp*-specific transcript and certain types of *Mbp* classic splice isoforms are indicative of mRNAs present in mature oligodendrocytes, while *Dm20* and *Cnp* transcripts are expressed during all stages of oligodendrocyte cell development (for review see Baumann and Pham-Dinh, 2001). As shown in Figure 1, mRNA levels for the examined myelin genes were consistently higher in Oli-neu cells compared to N20.1 cells. The *Dm20* splice variant was the predominant isoform expressed from the *Plp1* gene in both cell lines. The level of *Plp* and *Dm20* mRNA species combined was 7.5-fold higher in Oli-neu cells, while the level for the *Dm20* transcripts alone was 5.8-fold higher. Oli-neu cells demonstrated a 39-fold higher amount of *Mbp* classic transcripts than did N20.1 cells. The level was even higher (61-fold) for the major *Mbp* splice variants expressed in adult mice (encoding the 18.5- and 14-kDa

isoforms). The *Cnp* mRNA level was also elevated (8.5-fold) in Oli-neu cells compared to N20.1 cells. Because of the higher proportion of splice products expressed by mature oligodendrocytes (i.e. *Plp* and *Mbp* adult isoforms) in Oli-neu cells compared to N20.1 cells and the greater abundance of myelin gene transcripts overall in these cells, the cell lines may be at slightly different stages of maturation, with Oli-neu cells being further along the oligodendrocyte differentiation pathway.

Myelin protein levels are higher in Oli-neu cells compared to N20.1 cells

Western blot analysis was performed to compare the relative level of myelin proteins expressed in the N20.1 and Oli-neu cell lines. An extract prepared from the brain of a mouse at P35 of age was also included in the analysis for reference. As shown in Figure 2, DM20 protein was present in both cell lines, with Oli-neu cells containing nearly twice the amount of that in N20.1 cells. PLP was not discernable in either cell line, although it was readily detected (as expected) in the brain extract from a P35 mouse. The *Cnp* gene was also expressed at the protein level in both cell lines with Oli-neu cells possessing dramatically higher amounts of CNP (notice that the immunoreactive band in the lane containing 10 µg of total protein from Oli-neu cells is still appreciably more intense than that for N20.1 cells with 30 µg of total protein). However, neither cell line demonstrated *Mbp* gene expression at the protein level. This result was not due to a lack of reactivity with the anti-MBP antibody since multiple isoforms were detected using brain extract or purified protein (MBP). In the initial characterization of the N20.1 cell line (Verity et al., 1993), no MBP-immunoreactive material was detected when the cells were grown at either the permissive (34°C) or nonpermissive (39°C) temperature [the cell line was generated by infection of primary cultures of mouse brain oligodendrocytes with a retroviral construct that expresses a temperature-sensitive variant of SV40 T antigen]. However in subsequent studies (Paez et al. (2004,2005), MBP was detected by Western blot analysis when the cells were permitted to differentiate by switching the temperature to 39°C for 7 days. Yet we could not detect MBP protein in N20.1 cells maintained under conditions of proliferation (34°C) or in Oli-neu cells. These results are not entirely surprising since *Mbp* mRNAs are trafficked from the soma to distal processes, and then localize in the myelin compartment where they are translated (reviewed by Barbarese et al., 1999). It is possible that additional signals, such as those provided by axons, are required before *Mbp* gene products become readily apparent. Nevertheless our results, all told, suggest that Oli-neu cells are further along the maturation pathway than N20.1 cells.

Expression of *Plp1-lacZ* fusion genes in N20.1 and Oli-neu cells correlates with the developmental status of the cell lines

As shown in Figure 3, the mouse *Plp1* gene contains seven major exons and a relatively large first intron (>8 kb) that accounts for nearly half of the gene. Previous studies have demonstrated that intron 1 DNA contains multiple transcription regulatory elements that influence *Plp 1* gene expression; at least some of which are necessary to affect the dramatic rise in *Plp 1* gene activity during the active myelination period of CNS development (for review see Wight and Dobretsova, 2004). Deletion-transfection analysis with *Plp1-lacZ* fusion genes established that an enhancer (ASE) and two negative regulatory regions (red-bracketed areas of Fig. 3) are active in N20.1 cells. To test whether a 3-bp mutation in the ASE sequence which disrupts a putative AP-1 binding site could abolish its activity in N20.1 cells, cells were transfected with the *Plp1-lacZ* constructs illustrated in Figure 3. All constructs contain *Plp1* genomic DNA including the proximal 2.4 kb of 5' flanking, all of exon 1, and the first 37 bp of exon 2, which is used to drive expression of a *lacZ* reporter gene cassette. The PLP(+)Z construct also contains all 8140 bp of *Plp1* intron 1 DNA, while PLP(-)Z lacks the intron altogether. PLPΔ809-5807 is similar to PLP(+)Z except for missing *Plp1* intron 1 positions 809-5807, including the ASE sequence (positions

1093-1177); both negative regulatory regions were preserved in PLPΔ809-5807. The levels of expression obtained with these constructs (and others) in N20.1 cells are shown in Figure 4. Results are reported as the amount of β-gal activity relative to that for PLP(−)Z which was arbitrarily set at 100% (in N20.1 cells). The level of β-gal activity for PLP(+)Z (192%) was almost twice that of PLP(−)Z in N20.1 cells. However, deletion of only intron 1 positions 809-5807 (including the ASE) resulted in a significant decrease in β-gal activity (18% for PLPΔ809-5807), most likely due to effects mediated by the two negative regulatory present within the remaining intronic sequence, which of course are absent from PLP(−)Z. Insertion of just the ASE sequence (intron 1 positions 1093-1177) into the deletion-junction site of PLPΔ809-5807 in either the forward (F) or reverse (R) orientation was sufficient to fully 'rescue' the level of β-gal activity [compare PLPΔ809-5807 + ASE-F at 183% and PLPΔ809-5807 + ASE-R at 206% to PLP(+)Z at 192%]. However, incorporation of the sequence in conjunction with a 3-bp mutation (AAT→CGA; positions 1134–1136) which disrupts a putative AP-1 binding site failed to rescue β-gal activity when inserted in the forward (mutASE-F) or reverse (mutASE-R) direction, suggesting that these nucleotides are critical for the ASE to be active, at least in N20.1 cells.

Parallel transfection analysis (Fig. 5) was also performed in Oli-neu cells. Three notable differences were observed with transfection in Oli-neu cells compared to N20.1 cells. 1) Expression of the PLP(+)Z construct was considerably elevated (27-fold higher) over the level produced by PLP(−)Z. Therefore, transfection results in Oli-neu cells are reported as fold differences relative to PLP(−)Z, which was arbitrarily set at 1-fold. 2) The negative regulatory elements, within intron 1 that are active in N20.1 cells, do not appear to be operational in Oli-neu cells [compare PLPΔ809-5807 at 1.04-fold to PLP(−)Z at 1-fold]. [Inactivity of the negative regulatory elements in Oli-neu cells, coupled with enhanced potency of the ASE, suggests that Oli-neu cells may be further along the differentiation pathway than N20.1 cells.] 3) Even though insertion of the ASE sequence (in either orientation) in PLPΔ809-5807 was able to restore the level of β-gal activity back to that attained with PLP(+)Z, the 3-bp mutation disrupting an AP-1-like binding site did not hinder the rescue effort, suggesting that these nucleotides are not critical for ASE activity in Oli-neu cells.

DISCUSSION

Immortalized cell lines are a valuable tool that can be used to study molecular processes in oligodendrocytic cells such as gene regulation, signaling, and interaction with neurons (for reviews see Trotter, 1993; Bongarzone et al., 1996). They represent a homogenous pool of cells at a particular developmental stage with unlimited expansion potential, as opposed to primary cultured cells. Two oligodendroglial cell lines, generated by immortalization of primary cultures obtained from mouse brain, were characterized here in terms of their respective levels of myelin gene expression and responsiveness to an enhancer (ASE) located in intron 1 of the *Plp1* gene. The N20.1 and Oli-neu cell lines represent unique and important tools to discern the mechanisms for transcriptional regulation of myelin related genes. Maturation of myelinating oligodendrocytes *in vivo* is characterized by up-regulation of myelin genes such as *Mbp* and *Plp1* in preparation for deposition and assembly of the myelin sheath (reviewed by Miller, 2002). Hence it is critical to fully appreciate the developmental stage of a cell line when using it as a model to investigate mechanisms of myelin gene regulation.

Plp1 is a good model gene to investigate molecular mechanisms of myelin gene expression. The gene encodes the most abundant myelin protein in the CNS and its expression is coordinately regulated with other myelin genes (e.g. *Mbp*). Early in CNS development, expression of the *Dm20* splice variant precedes that for *Plp*. However as development

progresses, the level of *Plp* mRNA in oligodendrocytes supersedes that for *Dm20* (Gardinier et al., 1986; Sorg et al., 1987; LeVine et al., 1990). In the current study, the *Dm20* transcript was the major (if not only) splice isoform present in N20.1 and Oli-neu cells, suggesting that both cell lines are representative of oligodendrocytes at an early developmental stage. Although quantification of mRNA levels by RT-PCR can vary among different primer/probe sets, results in Figure 1 suggest that the *Plp*-specific species may actually be formed (to a slight degree) in Oli-neu cells [notice that the relative level for *Plp/Dm20* transcripts combined was slightly higher than that for *Dm20* mRNA alone in Oli-neu cells, but the reverse was observed in N20.1 cells]. Whatever the case may be, the overall level of transcripts derived from the *Plp1* gene is higher in Oli-neu cells compared to N20.1 cells (Fig. 1).

Like the *Plp1* gene, expression of the *Mbp* gene is temporally regulated in brain, with peak mRNA levels being attained several days earlier (P18 vs. P21) in mice (Sorg et al., 1987). Due to alternative splicing, six 'classic' MBP products are generated in mouse (Barbarese et al., 1978; de Ferra et al., 1985; Harauz et al., 2004). Exon 2-containing isoforms (21.5, 20.5, and 17.22 kDa) are expressed at higher levels early in myelination as well as in immature oligodendrocytes in culture (Barbarese et al., 1978; de Vries et al., 1997), whereas the remaining isoforms (18.5, 17.24, 14 kDa) are observed in adult myelin and mature oligodendrocytes (reviewed by Boggs, 2006). qRT-PCR analysis (Fig. 1) with a probe that detects the entire spectrum of *Mbp* (classic) transcripts demonstrated that *Mbp* mRNA levels were much higher in Oli-neu cells compared to N20.1 cells. The same was true when the 'adult' transcripts were measured, exclusively. While the amount of mature splice variants was significantly lower in comparison to the total level of *Mbp* transcripts in both cell lines, the relative proportion of mature transcripts was higher in Oli-neu cells, signifying that these cells are at a slightly later stage in development than N20.1 cells. The low levels of mature *Mbp* mRNAs in N20.1 cells are consistent with an earlier study (Verity et al., 1993) which showed that the cell line primarily generates transcripts encoding the 17 and 21.5 kDa isoforms and, to a much lower extent, the adult 18.5 kDa isoform.

Consistently, mRNA levels for another myelin gene (*Cnp*) were also higher in Oli-neu cells. Expression of the *Cnp* gene is developmentally regulated. In the rodent brain, *Cnp* mRNA can be detected as early as E15, with peak levels occurring at P15 (Kanfer et al., 1989). Taken as a whole, the qRT-PCR results suggest that both the Oli-neu and N20.1 cell lines are at an early stage of maturation, resembling that observed *in vivo* prior to myelination (Kanfer et al., 1989), with Oli-neu cells being slightly more advanced.

In general, the level of myelin proteins in N20.1 and Oli-neu cells, as measured by Western blot analysis (Fig. 2), coincided with their respective mRNA levels. *Plp1* gene products could be discerned in N20.1 cells, unlike in the initial characterization of the cell line (Verity et al., 1993). However, only the DM20 isoform was detected, with Oli-neu cells having a greater amount of the protein. Although the results from qRT-PCR analysis suggest that a minute quantity of the *Plp*-specific transcript may be formed in Oli-neu cells (but not N20.1 cells), the amount of PLP (if produced) was below the limits of detection. Furthermore, CNP protein was detected in both cell lines, with much higher levels being present in Oli-neu cells.

We were unable to detect MBP polypeptides by Western blot analysis in either cell line, even though immunoreactive bands were clearly observed with brain lysates or purified MBP isoforms. The lack of MBP in N20.1 cells is consistent with a previous report (Verity et al., 1993) which identified *Mbp* transcripts, but not the protein, in this cell line. *Mbp* gene expression has been shown to be regulated at the post-transcriptional level by a variety of factors. For example, the Quaking RNA-binding protein (QKI) has been proposed to

regulate *Mbp* gene expression via mRNA stability, subcellular distribution, and alternative splicing (reviewed by Zearfoss et al., 2008), while hnRNP A2 is important for the intracellular trafficking of *Mbp* mRNA to the myelin compartment where it is then translated (reviewed by Barbarese et al., 1999). Presumably these or other issues, such as the lack of axonal signals, thwart the synthesis of MBP in the cell lines used in the current study.

Plp1 gene activity is regulated via multiple regulatory elements, including several located in the first intron (Li et al., 2002). In N20.1 cells, the ASE appears to be the sole positive regulatory element within *Plp1* intron 1 DNA. It functions as an enhancer (Dobretsova et al., 2000); however it needs to be in the confines of the intron in order to work (Meng et al., 2005). While the full ASE sequence functions in an orientation-independent manner, deletion of distal sequences (approximately latter one-third) necessitates that it be in the native direction to be effective (Dobretsova et al., 2004). Moreover, a 3-bp substitution in the truncated ASE sequence (disrupting an AP-1-like binding site) precludes any enhancer activity. The effect of the mutation in context of the full-length ASE sequence was examined in the present study. When tested in N20.1 cells, the mutation negated the ability of the full-length ASE sequence to rescue β -gal activity in either orientation (Fig. 4). Surprisingly, the mutation did not affect the ability of the full-length ASE sequence to rescue β -gal activity in Oli-neu cells (Fig. 5). Taken together, the transfection results suggest that: (i) the ASE is active in Oli-neu cells. In fact the relative strength of the ASE seems to be more potent in the Oli-neu cells than N20.1 cells. (ii) Because the AP-1-like binding site in the ASE is critical for enhancer activity in N20.1 but not Oli-neu cells, there must be differences between the repertoire of nuclear factors (or divergence in the amount or chemical modification of pertinent factors) in these cells, conceivably due to the lines being at different developmental stages.

In summary, the transfection results along with the observed differences in the pattern and quantity of myelin gene expression support the hypothesis that Oli-neu cells are slightly further along in the oligodendroglial maturation process than N20.1 cells when maintained under the conditions specified here. Because the level of CNP was highest among the myelin proteins examined, both cells lines are likely representative of oligodendrocytes at an immature state [CNP is expressed in both immature and mature oligodendrocytes (Amur-Umarjee et al., 1990)]. Thus, these cell lines may afford an opportunity in which to investigate the regulation of myelin gene expression at slightly different developmental stages.

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Fig. 1.

Real-time RT-PCR analysis in N20.1 and Oli-neu cells. mRNA levels for the *Plp1*, *Mbp*, and *Cnp* genes were determined using a standard curve generated with cDNA prepared from N20.1 cells, and are reported as amounts (ng) relative to that for β -actin. *Plp/Dm20* depicts the combined level of (classic) transcripts produced by the *Plp1* gene, while the adjacent column represents the level for the *Dm20* splice variant, exclusively. *Mbp* denotes all splice isoforms, while only the splice variants lacking exon 2 were measured in the *Mbp* (adult) column. These results demonstrate that Oli-neu cells have higher mRNA levels for the myelin genes tested than do N20.1 cells.

Fig. 2.

Oli-neu cells contain higher levels of myelin proteins than N20.1 cells. Cell lysates were prepared from N20.1 and Oli-neu cells and subsequently analyzed for the presence of PLP/DM20, CNP, and MBP proteins by Western blot analysis. An extract prepared from the brain of a mouse at P35 of age, as well as purified MBP, were also included as controls. Results show that the levels for DM20 and CNP are higher in Oli-neu cells than N20.1 cells [graph to the right shows the relative levels of DM20 and CNP in the lanes loaded with 10 and 30 μ g of total protein, respectively, as determined by image analysis]. MBP was not detected in either cell line. β -actin was included as a control for amounts of proteins loaded in the lanes containing extract.

Fig. 3.

Schema of mouse *Plp1* gene and *Plp1-lacZ* constructs. The mouse *Plp1* gene spans 15.6 kb of DNA from the beginning of exon 1 to the end of exon 7, and contains a relatively large (>8 kb) first intron. Only the major exons (boxes) are indicated. Exon 3 contains an internal splice donor site. DM20 protein is generated from transcripts that utilize the internal 5'-splice site; PLP-specific residues are encoded by the sequence depicted in the gray box. The regulatory elements in intron 1 DNA that are effective in N20.1 cells are designated by a star (ASE positive regulatory element) and two bracketed regions, which contain negative regulatory elements (Dobretsova and Wight, 1999). The *Plp1-lacZ* constructs used in the current study contain *Plp1* genomic DNA from the proximal 2.4 kb of 5'-flanking DNA, downstream to the first 37 bp of exon 2. PLP(+)Z contains all of *Plp1* intron 1 DNA, while PLP(-)Z is missing the entire intron. The PLPΔ809-5807 construct was generated from PLP(+)Z by excision of the indicated PstI (P) fragments. It lacks *Plp1* intron 1 DNA from position 809 to 5807 based on numbering the intron from positions 1 to 8140 (Wight and Dobretsova, 1997).

Fig. 4.

Transfection analysis of *Plp1-lacZ* constructs in N20.1 cells. **Left:** Cartoon depicting the structure of the *Plp1-lacZ* constructs. Black boxes represent *Plp1* exonic sequence, while solid lines indicate *Plp1* 5'-flanking DNA or intron 1 sequence. Dotted lines correspond to *Plp1* intron 1 sequence that is missing from the constructs. The open box marked ASE (not drawn to scale) denotes incorporation of the ASE sequence (intron 1 positions 1093-1177) into the deletion-junction site of PLP Δ 809-5807 in the forward (F) or reverse (R) direction. In some cases, a mutated (mut) ASE sequence (ASE*) was inserted. See Fig. 3 for further details about the constructs. **Right:** N20.1 cells were transiently cotransfected with equimolar amounts of *Plp1-lacZ* constructs along with a set amount of RSVL to correct for differences in transfection efficiency. Transfection results are presented as the mean $\pm$ SD of β -gal activity ($n \geq 6$) relative to that obtained for PLP(-)*Z*, which was arbitrarily set at 100% in every experiment. *Significant difference ($P < 0.05$) between PLP(+)*Z* and other constructs by one-way ANOVA with Bonferroni post-hoc analysis.

Fig. 5.

Transfection analysis of *Plp1-lacZ* constructs in Oli-neu cells. Oli-neu cells were transiently cotransfected with equimolar amounts of *Plp1-lacZ* constructs along with a set amount of RSVL to correct for differences in transfection efficiency. See Fig. 4 for details regarding the *Plp1-lacZ* constructs. Transfection results represent the fold change of the mean $\pm$ SD in β -gal activity ($n \geq 6$) relative to that obtained with PLP(-)*Z*, which was arbitrarily set at 1 for each experiment. *Significant difference ($P < 0.05$) between PLP(+)*Z* and other constructs by one-way ANOVA with Bonferroni post-hoc analysis.