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Leydig cells express the myelin proteolipid protein gene and incorporate a new alternatively spliced exon

Shenyang Li [a,1], Brian T. Greuel [b,1], Fanxue Meng [a], Glauber B. Pereira [a], Adria Pitts [b], Anna Dobretsova [a], Patricia A. Wight [a,*]

[a] Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Mail Slot #750, 4301 W. Markham St., Little Rock, AR 72205, USA
[b] Department of Biology, John Brown University, Siloam Springs, AR 72761, USA

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ABSTRACT

Although the myelin proteolipid protein gene (*Plp1*) is highly expressed in the central nervous system encoding the most abundant myelin protein in oligodendrocytes, it is also expressed in other tissues, including testis. Transgenic studies with mice that harbor *Plp1–lacZ* fusion genes suggest that Leydig cells are the source of *Plp1* gene expression in testis. However, virtually nothing is known about *Plp1* gene regulation in Leydig cells, which is the focus of this study. The first intron contains both positive and negative regulatory elements that are important in regulating *Plp1* gene expression in oligodendrocytes. To test whether these elements are functional in Leydig cells, a battery of *Plp1–lacZ* fusion genes with partial deletion of *Plp1* intron 1 sequence was transfected into the mouse Leydig cell line, TM3. Results presented here suggest that an enhancer, which is very potent in oligodendrocytes, is only nominally active in TM3 cells. The intron also contains several negative regulatory elements that are operative in TM3 cells. Moreover a new exon (exon 1.2) was identified within the first 'intron' resulting in novel splice variants in TM3 cells. Western blot analysis suggests that these splice variants, along with those containing another alternatively spliced exon (exon 1.1) derived from intron 1 sequence, give rise to multiple *Plp1* gene products in the mouse testis.

1. Introduction

While the myelin proteolipid protein gene (*Plp1*) is highly expressed in myelinating cells (oligodendrocytes) of the brain and spinal cord, it is also expressed to a lesser degree in a variety of non-neural tissues (Wight and Dobretsova, 2004, and references therein), including testis. A *Plp1–lacZ* transgene [PLP(+)Z], which contains mouse *Plp1* genomic DNA extending from the proximal 2.4 kb of 5′-flanking DNA downstream to the first 37 bp of exon 2, is sufficient to drive *lacZ* reporter gene expression in a spatial manner consistent with endogenous *Plp1* expression, as well as developmentally in brain (Wight et al., 1993). Yet a related transgene, PLP(−)Z, which is identical to PLP(+)Z except for the absence of *Plp1* intron 1 DNA, is expressed to much lower levels in brain, where its developmental pattern is exceedingly attenuated (Li et al., 2002b). In fact expression of the PLP(−)Z transgene was so low in brain, that on initial inspection, the lines would have been characterized as non-expressing if not for a fair amount of expression in testis. Histological analysis showed that Leydig cells were the source of transgene expression in the testis (Li et al., 2002b). However, regulation of *Plp1* gene expression in Leydig cells has not been elucidated until now.

Previous studies have shown that the first intron of the *Plp1* gene contains several regulatory elements that are important modulators of expression in other cell types. In oligodendrocytes, there is a single positive regulatory element that resides between intron 1 positions 1083 and 1177, which can override repression mediated by a couple of general negative regulatory elements located elsewhere in the intron (Dobretsova and Wight, 1999). Because the positive regulatory element also displays enhancer-like qualities [i.e., orientation-independent; activity is increased with multiple copies (Dobretsova et al., 2000); can activate a heterologous promoter (Meng et al., 2005)] it was named ASE for antisilencer/enhancer. However the ASE does not function in a liver cell line (+/+ Li; ATCC, Rockville MD, USA, catalog number CRL-6467), although the general negative regulatory elements are effective in addition to a cell-type-specific negative regulatory element located near the 3′ end of intron 1 (Li et al., 2002a). In the present study we have determined the influence of these regulatory elements on *Plp1* gene expression in a Leydig cell line (TM3). [The TM3 clone was derived from primary cultures of Leydig cell-enriched preparations from normal testes of 11- to 13-day-old

Abbreviations: ASE, antisilencer enhancer; βGal, β-galactosidase; bp, base pairs; nt, nucleotides; PLP, proteolipid protein; RLU, relative light units; RSV, Rous sarcoma virus; RT, reverse transcription; *tsp*, transcription start point; u, units.

* Corresponding author. Tel.: +1 501 686 5366; fax: +1 501 296 1469.
E-mail address: pwight@uams.edu (P.A. Wight).
[1] These authors contributed equally to this work.

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BALB/c mice and retains many of the functions characteristic of the cell type *in vivo* (Mather, 1980).] As a corollary to these studies, two alternatively spliced exons were identified from what is classically defined as *Plp1* intron 1 DNA. One of these, exon 1.1, has been described previously as an additional exon that is included in some of the transcripts from oligodendrocytes, neurons, and lymphoid cells (Bongarzone et al., 1999; Feng et al., 2003; Jacobs et al., 2003). However identification of the other exon, denoted here as exon 1.2, is novel. Inclusion of one or both of these exons, coupled with additional alternative splicing due to the presence of two donor splice sites in exon 3 (Nave et al., 1987), leads to a variety of splice variants in Leydig cells, which ultimately results in the expression of multiple *Plp1* gene products in testis.

2. Materials and methods

2.1. Cell culture

The mouse Leydig cell line, TM3 (ATCC, catalog number CRL-1714), was grown at 37 °C in a 1:1 mixture of Ham's F-12/Dulbecco's modified Eagle's medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 1.2 g/L sodium bicarbonate, 15 mM HEPES, 4.5 g/L glucose, 2.5% fetal bovine serum (Intergen, Purchase, NY, USA), 5% horse serum (HyClone, Logan, UT, USA), and maintained in an atmosphere of 5% CO_2. The mouse liver cell line, +/+ Li (ATCC), was grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 3.7 g/L sodium bicarbonate, 10% fetal bovine serum (Intergen), and maintained in an atmosphere of 10% CO_2. The mouse immortalized oligodendrocyte cell line N20.1 (Verity et al., 1993) was grown at 34 °C in a 1:1 mixture of Ham's F-12/Dulbecco's modified Eagle's low glucose medium (Irvine Scientific) supplemented with 1.2 g/L sodium bicarbonate, 3.75 g/L HEPES, 5.75 g/L glucose, 100 μg/ml G-418, 10% fetal bovine serum (Intergen), and maintained in an atmosphere of 5% CO_2.

2.2. Plp1–lacZ plasmids

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 sequences extending from the proximal 2.4 kb of 5′-flanking DNA, downstream to the first 37 bp of exon 2, which are used to drive expression of a *lacZ* reporter gene cassette. The PLP(−)Z construct (Wight and Dobretsova, 1997) is missing all of *Plp1* intron 1 DNA, while partial deletion constructs contain only a portion of the intron and were named accordingly. For instance PLPΔ809–5807 (Dobretsova and Wight, 1999) is missing *Plp1* intron 1 DNA from positions 809 to 5807 based upon numbering the intron from positions 1 to 8140 (Wight and Dobretsova, 1997). Plasmids PLPΔ809–5807 + F(I–V) and PLPΔ809–5807 + R(I–V) contain *Plp1* intron 1 DNA positions 1083–1177 (I–V) inserted into the deletion junction site of PLPΔ809–5807 in the native (forward; F) or reverse (R) orientation, respectively (Dobretsova et al., 2000).

The pPUR/PLPΔ809–5807 plasmid (Dobretsova et al., 2000) was used as the foundation from which to generate pPUR/PLP(+)Z and pPUR/PLP(−)Z. The plasmid contains *Plp1–lacZ* sequences from PLPΔ809–5807 cloned into the pPUR vector (Clontech Laboratories, Palo Alto, CA, USA) which allows for coexpression of a puromycin-resistance selectable marker. pPUR/PLP(+)Z was produced by digestion of PLP(+)Z and pPUR/PLPΔ809–5807 with ApaI and NotI and subsequent ligation of the appropriate fragments to yield a plasmid that is essentially PLP(+)Z in the background of the pPUR vector. The pPUR/PLP(−)Z plasmid was generated similarly from PLP(−)Z and pPURΔ809–5807 and is identical to pPUR/PLP(+)Z, except for the lack of *Plp1* intron 1 DNA.

2.3. Transfection analysis

TM3 cells were transiently transfected with equimolar amounts of various *Plp1–lacZ* fusion genes (in addition to an RSV-luciferase plasmid to control for differences in transfection efficiency) using the Lipofectamine Reagent as previously described (Dobretsova and Wight, 1999). Cell lysates were prepared approximately 31 h post DNA addition and reporter gene activities determined by chemiluminescent detection as previously described (Dobretsova and Wight, 1999). Results have been corrected for differences in transfection efficiency and represent the mean ± SD of βGal activity relative to that obtained for PLP(−)Z transfected cells (arbitrarily set at 100% in every experiment) from three or more independent experiments.

Stable transfections were performed similar to the methods used for transient transfection. The day prior to transfection, cells were seeded in 6-well microplates at the following densities: 2.0×10^5 (TM3), 2.4×10^5 (+/+ Li), and 2.8×10^5 (N20.1) per 35-mm well. Cells were transfected with equimolar amounts of pPUR/PLP(+)Z or pPUR/PLP(−)Z linearized with AseI, which cleaves the plasmid at a single site in the vector backbone. Cells were grown in the appropriate growth medium for 48 h post-transfection and then switched to medium containing puromycin (Clontech): 4 μg/ml (TM3); 1.5 μg/ml (+/+ Li); and 3 μg/ml (N20.1). Puromycin-resistant clones were picked, pooled (200–300 individual colonies per pool), and expanded in culture. Lysates were prepared from a portion of each pool, and βGal activities measured as for the transient transfections. The protein concentration of lysates was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Results are presented as βGal activity in relative light units (RLU) per microgram of total protein.

2.4. Ribonuclease protection assays (RPA)

Total RNA was isolated from a portion of each expanded pool of stably transfected cells (or untransfected cells) using the Trizol Reagent (Invitrogen) as recommended by the supplier. RPA analysis was performed as previously described (Li et al., 2002b) with the RPA III Kit (Ambion, Austin, TX, USA). Briefly, 25 μg of total RNA was hybridized overnight at 43.5 °C with ^{32}P-labeled riboprobes. The full-length *Plp1–lacZ* antisense riboprobe is 387 nt in size and includes the distal half of *Plp1* exon 1 sequence along with the first 37 nt of exon 2, in addition to sequence complementary to the *lacZ* expression cassette (Li et al., 2002b). A 304 nt antisense riboprobe generated from the pTRI-β-actin-Mouse Template (Ambion) was also included to assess the endogenous level of β-actin mRNA, as an internal control. After hybridization, single-stranded RNA was removed using a mixture of RNase A/T1 and the remaining double-stranded RNA (protected fragments) fractionated on a 6% acrylamide–8 M urea gel. Dried gels were first analyzed on a 445 SI PhosphorImager with ImageQuantNT (Molecular Dynamics, Sunnyvale, CA, USA) and then subjected to autoradiography. Hybridization of 'classic' *Plp1–lacZ* transcripts (i.e., does not contain any *Plp1* intron 1 sequence such as exon 1.1) results in protection of 335 nt of the riboprobe, while hybridization to transcripts containing alternatively spliced exon(s) derived from *Plp* intron 1 results in protection of a slightly smaller stretch (258 nt). Hybridization of mRNA to the β-actin riboprobe results in the protection of a 245 nt expanse.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from TM3 cells (or adult mouse testis) as described earlier and treated with RQ1 RNase free DNase (1 u/μg) (Promega, Madison, WI, USA) for 1 h to eliminate residual DNA contamination. Reverse transcription (RT) was performed at 42 °C for 50 min in a total volume of 20 μl using 0.5 μg of oligo$(dT)_{12-18}$ primer and 200 u of Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen). The reaction was halted by heating at 70 °C for 15 min