

Chapter 2

Identification of a Novel Family of Oligodendrocyte Lineage-Specific Basic Helix-Loop-Helix Transcription Factors

Identification of a Novel Family of Oligodendrocyte Lineage-Specific Basic Helix–Loop–Helix Transcription Factors

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Summary

Basic helix–loop–helix (bHLH) transcription factors have been identified for neurons and their precursors but not for glial cells. We have identified two bHLH factors, *Oligo1* and *Oligo2*, that are specifically expressed in zones of neuroepithelium from which oligodendrocyte precursors emerge, as well as in the precursors themselves. Expression of *Oligo2* in the spinal cord precedes that of platelet-derived growth factor receptor α (PDGFR α), the earliest known marker of oligodendrocyte precursors, by several days. Ectopic expression of *Oligo2* in vivo causes ectopic expression of *Sox10*, an HMG-box transcription factor expressed in oligodendrocyte and other glial precursors. These data identify *Oligo* genes as the earliest known markers of oligodendrocyte lineage determination and suggest they play a causal role in this process.

Introduction

Oligodendrocytes are the myelinating glial cells of the central nervous system (CNS). The development of oligodendrocytes is of considerable importance to an understanding of both neural development and the pathogenesis of demyelinating diseases, such as multiple sclerosis. Oligodendrocytes differentiate from bipolar, migratory precursor cells that arise from specific zones of neuroepithelium and then disperse throughout developing gray matter (reviewed by Miller, 1996). A great deal is known about the control of proliferation, survival, and differentiation of oligodendrocyte precursors (reviewed by Raff, 1989; Barres and Raff, 1994). In contrast, much less is known about the factors that control the initial commitment of neuroepithelial cells to the oligodendrocyte lineage.

In the developing spinal cord, oligodendrocyte precursors identified by expression of platelet-derived growth factor receptor α (PDGFR α) (Pringle and Richardson, 1993; Hall et al., 1996) and other differentiation markers (Yu et al., 1994; Ono et al., 1995; Timsit et al., 1995) are generated from a narrow zone in the ventral region of the neuroepithelium (Warf et al., 1991; Pringle et al., 1998; reviewed by Miller, 1996). These precursors begin to emerge in mouse embryos on embryonic day

12.5 (E12.5) (Calver et al., 1998), after the period of motor neuron production has ended (Nornes and Das, 1974; Altman and Bayer, 1984). The generation of oligodendrocyte precursors from this zone requires ventralization of the neural tube by notochord- and floor plate-derived Sonic hedgehog (Shh) (Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996; Orentas et al., 1999). Lineage tracing studies in vivo have shown that oligodendrocytes share a common precursor with neurons and astrocytes in the spinal cord (Leber et al., 1990), a result confirmed by clonal analyses of multipotent neural stem and progenitor cells in vitro (Williams et al., 1991; Temple and Davis, 1994). However, the time at which motor neuron- and oligodendrocyte-producing lineages diverge in the spinal cord is not yet clear. These observations raise the related issues of how the timing and spatial restriction of oligodendrocyte production in the spinal cord are achieved and its relationship to the generation of motor neurons and other ventral neuronal subtypes (Tanabe and Jessell, 1996).

These questions have been difficult to address because it is not yet clear when ventricular zone cells first become determined for an oligodendrocyte fate. The earliest known marker for these cells is PDGFR α (reviewed by Miller, 1996); however, it is possible that determinative events for the oligodendrocyte lineage have already occurred before this receptor is first expressed. Transcription factors have proven to be useful early markers of lineage determination in other regions of the vertebrate nervous system (Lo et al., 1991; Ericson et al., 1992; reviewed by Tanabe and Jessell, 1996). However, there are relatively few transcription factors that mark the earliest stages of oligodendrocyte development (Collarini et al., 1992).

Basic helix–loop–helix (bHLH) proteins are a family of transcriptional regulators that play a key role in cell-type determination in a variety of tissues and organisms (reviewed by Garrell and Campuzano, 1991). In the nervous system, a large number of bHLH factors have been identified and studied genetically (reviewed by Kagayama and Nakanishi, 1997; Lee, 1997). These factors include vertebrate homologs of *Drosophila* proneural genes, such as *Mash1*, the *Neurogenins*, and *NeuroD* (Johnson et al., 1990; Lee et al., 1995; Naya et al., 1995; Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996). For the most part, these genes appear to be involved in neuronal rather than glial development; indeed, no bHLH factor has yet been identified that is specifically expressed in glial precursors in either the CNS or the peripheral nervous system (PNS). The general importance of bHLH factors in cell-type determination suggested, however, that the identification of such factors for glial cells would be an important first step in understanding the early events underlying the determination of glial lineages.

Here, we describe the identification and preliminary characterization of two novel bHLH factors, called *Oligo1* and *Oligo2*, that appear to be specifically expressed in oligodendrocyte precursors throughout the CNS. These genes are also expressed in the restricted

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A

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m.Oligo1      M L R P Q R P G D V Q L Q A S L Y E L V C Y R O P P I S S S S S S S S S T A S L L P
m.Oligo2     M D S D A S L V S S R P S S S P E P D D L F L P A R S K G C S S S S G . F T G G T V S S S S T P S D C P P

m.Oligo1     K P A R E K A E A P L A E P R G P A P E S G G . . . . . A R A D A K E . . .
m.Oligo2     E L S S E L R G A M G A S G A H P G D K L G G G G F K S S S S S T S S S T S S A A T S S T K K D K K

m.Oligo1     . . . E Q Q Q Q O Q L R R K I N S R E R K R M Q D L N L A M D A L R E V I L P Y S A A H C O G A P G R
m.Oligo2     Q M T E P E L O Q L R L K I N S R F R K R M H D I N I A M D G L R E V M P . . . . . Y A H G P S V R

m.Oligo1     K L S K I A T L L L A R N Y I L L L G S S L Q E L R R A L G D G A G P A A P R L L L A G L P L L A A
m.Oligo2     K L S K I A T L L L A R N Y I L M L T N S L E E M K R L V S E I Y G . . . . . G H H A G F H P S A C

m.Oligo1     A P G S V L L A P G A V G P P E T L R P T K Y L S - A L D E P P C G G O F S L P A G G A A G S P G L C S
m.Oligo2     G G . . . . L A H S A P I P T A T A H P A A A A H A A H H . P A V H H P I L P P A A A A A A A A A A A A A

m.Oligo1     C A V C K F P H L V P A G L G L A A V Q A O F S K
m.Oligo2     A A A V S S A S L P . . . G S G L S S V
    
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B

	← Basic →	← Helix1 →	← Loop →	← Helix2 →
m.Oligo1	L R R K I N S R E R K R M Q D L N L A M D A L R E V I L P Y S A A H C O G A P G R	K L S K I A T L L L A R N Y I L L		
m.Oligo2	L R L K I N S R E R K R M H D L N I A M D G L R E V M P Y A H G P S V R	K L S K I A T L L L A R N Y I L M L		
r.Kw8	R R Q K A N A R E R N R M H D L N A A L D A L R S V L P S F P D D Q	K L S K I E T L R L A K N Y I W A L		
m.Ngn1	R R V K A N D R E R N R M H N L N A A L D A L R S V L P S F P D D T	K L S K I E T L R A Y N Y I W A L		
m.NeuroD	R R R K A N A R E R N R M H G L N A A L D N L R K V V P C Y S K T O	K L S K I E T L R L A K N Y I W A L		
m.Math2	R R Q E A N A R E R N R M H G L N D A L D N L R K V V P C Y S K T O	K L S K I E T L R L A K N Y I W A L		
m.Mash1	A V A R A N E R E R N R V K L V N L G F A T L R E H V P N G A A N K	M S K V E T L R S A V Q Y I R A L		
m.Dhand	R R G T A N R K E R R R T Q S I N S A F A E L R E C I P N V P A D T	K L S K I K T L R L A T S Y I A Y L		
m.Myogenin	R R R A A T L R E K R R L K K V N E A F E A L K R S T L L N P N Q R	L P K V E T L R S A I Q Y I E R L		

C

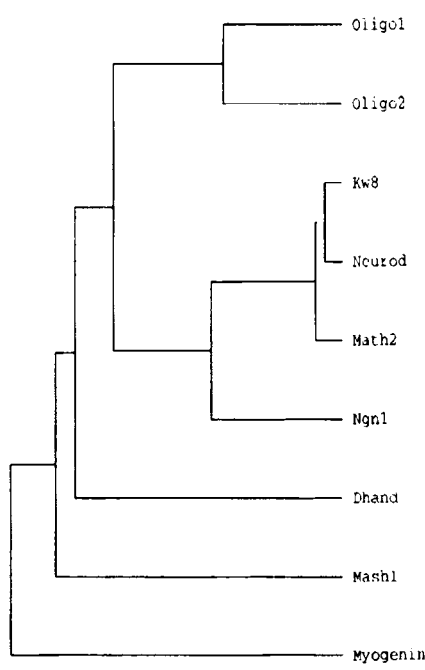


Figure 1. Sequence of Murine Oligo1 and Oligo2 and Comparison with Other bHLH Factors
 (A) Deduced amino acid sequences of *Mus musculus* Oligo1 and Oligo2. Identical amino acids are boxed. Arrowheads indicate the bHLH domain.
 (B) Alignment of the bHLH domains of m.Oligo1 and m.Oligo2 with those of other bHLH factors. Note that while the bHLH domains of Oligo1

zones of neuroepithelium from which oligodendrocyte precursors emerge but are detected considerably earlier than is PDGFR α . Ectopic expression of *Oligo2* in vivo induced ectopic expression of *Sox10*, an HMG-box protein that is another early transcriptional marker of glial lineages (Kuhlbrodt et al., 1998) and that has been functionally implicated in myelination in humans (Inoue et al., 1999). Taken together, these data suggest that *Oligo1* and *Oligo2* are bHLH determination and/or differentiation factors for myelinating glial cells in the CNS.

Results

Identification of *Oligo1* and *Oligo2* by Database Screening

We initially identified *Oligo1* and *Oligo2* in a database screen for as yet uncharacterized bHLH factors that might be expressed in the nervous system. *Oligo1* and *Oligo2* sequences were identified as human genomic sequences present on a single bacterial artificial chromosome (BAC) clone derived from chromosome 21q (see Experimental Procedures). A sequence corresponding to human *Oligo2*, called RK17, was also deposited by Kuroda et al. (GenBank accession number Q13516). Preliminary in situ hybridization data for these genes were obtained using murine probes generated by PCR amplification of mouse genomic DNA with primers based on the human sequences. These data suggested that *Oligo1* and *Oligo2* might be expressed in oligodendrocyte-generative zones of the spinal cord (see below).

To obtain the entire coding sequences of *Oligo1* and *Oligo2*, we took advantage of the fact that the coding sequences of many bHLH factors are contained within a single exon (e.g., see Guillemot et al., 1993; Fode et al., 1998; Ma et al., 1998), and isolated and sequenced murine genomic clones encoding these genes. The deduced amino acid sequences of *Oligo1* and *Oligo2* are shown in Figure 1A. The two sequences are virtually identical in the bHLH domain, except for the loop region, which is significantly divergent (78% overall amino acid identity in the bHLH domain; Figure 1B). Outside of the bHLH region, however, the two sequences are highly divergent (Figure 1A). Both sequences contain strings of repeated amino acids outside of their bHLH domains, like many other transcription factors. *Oligo1* and *Oligo2* have been independently isolated by Rowitch and coworkers in the rat, for which they have been called *Olg-1* and *Olg-2*, respectively (Lu et al., 2000 [this issue of *Neuron*]).

Alignment of the *Oligo1* and *Oligo2* bHLH domains with those of other bHLH factors indicates that these proteins constitute a distinct subfamily (Figures 1B and 1C). This subfamily is more closely related to vertebrate bHLH factors homologous to *Drosophila* ATONAL (Jarman et al., 1993), such as NeuroD (Lee et al., 1995) and

Neurogenin 1 (Ma et al., 1996) than it is to other bHLH factors expressed in the developing nervous system, such as Mash1 (Johnson et al., 1990) or dHAND (Srivastava et al., 1995) (Figure 1C). Interestingly, all of these neural bHLH factors were more related to each other than to Myogenin (Edmondson and Olson, 1989), a representative of the muscle-specific subfamily of bHLH factors (Molkentin and Olson, 1996; Yun and Wold, 1996) (Figure 1C).

Oligo2 and *Oligo1* Are Specifically and Sequentially Expressed in the Region of the Spinal Cord Neuroepithelium from which Oligodendrocytes Emerge

In situ hybridization analysis of *Oligo1* and *Oligo2* revealed that they are expressed in the ventral spinal cord as early as E9.5 (data not shown). *Oligo2* is expressed much more strongly than *Oligo1* is at this stage. Both genes appear to be expressed in the ventral third of the developing neural tube but not in the floor plate (Q. Z. and D. J. A., unpublished data). Over the next few days, *Oligo2* expression declines in intensity and becomes progressively restricted to a very narrow band within the ventral neuroepithelium of the spinal cord (Figure 2A, arrowhead; data not shown). By E12.0, expression of *Oligo2* in this region has increased (Figure 2E). *Oligo1* expression declines to undetectable levels by E10.5 and reappears in the same narrow zone as *Oligo2* at E12.0 (Figure 2G). Thus, both genes appear to have early and late phases of expression within the ventral spinal cord. Double-labeling experiments confirmed that these two genes are expressed in the same zone of the neuroepithelium at E12.0 (Figures 3A and 3D).

The site of expression of these genes was highly reminiscent of the region from which oligodendrocyte precursors have been reported to emerge in the spinal cord (Pringle and Richardson, 1993). We therefore compared the expression of *Oligo2* and *Oligo1* to that of several other oligodendrocyte precursor markers. A comparison to PDGFR α indicated that the genes were indeed expressed in an overlapping if not identical domain of the neuroepithelium, except that the bHLH factors were expressed earlier than was the growth factor receptor. *Oligo2* is expressed about 1–1.5 days before PDGFR α mRNA is detectable (Figures 2A and 2L), while *Oligo1* is detected about a day earlier than is PDGFR α (Figures 2G, arrowhead, and 2L, arrows). Double-label in situ hybridization experiments confirmed that PDGFR α and *Oligo* genes are likely coexpressed in the same cells (Figures 3C and 3F, arrows; data not shown). By E14.0, *Oligo1*⁺ and *Oligo2*⁺ cells had begun to disperse through the gray matter (Figures 2M and 2O, arrow), in a ventral-to-dorsal manner similar to that taken by PDGFR α ⁺ cells (Figure 2P). At this stage, some *Oligo1*⁺ and *Oligo2*⁺ cells still remained in the ventricular zone (Figures 2M

and *Oligo2* are closely related (B), there is little if any sequence homology N- or C-terminal to this region (A) Abbreviations: r, rat, and m, mouse.

(C) Dendrogram of *Oligo1* and *Oligo2* bHLH sequences in relation to other bHLH factors. *Oligo1* and *Oligo2* form a subfamily distinct from the other bHLH factors, and the neural bHLH factors as a group are distinct from the myogenic bHLH factors represented by Myogenin. Sources of the indicated sequences are r.KW8 (Kume et al., 1996), NeuroD (Lee et al., 1995), Ngn1 (Ma et al., 1996), Math2 (Bartholom  and Nave, 1994; Shimizu et al., 1995), Mash1 (Franco del Amo et al., 1993; Guillemot and Joyner, 1993), dHAND (Srivastava et al., 1995), and Myogenin (Edmondson and Olson, 1989). Sequence alignments were performed using the ClustalW program provided with MacVector software, and the dendrogram was generated using the Pileup Program (GCG).

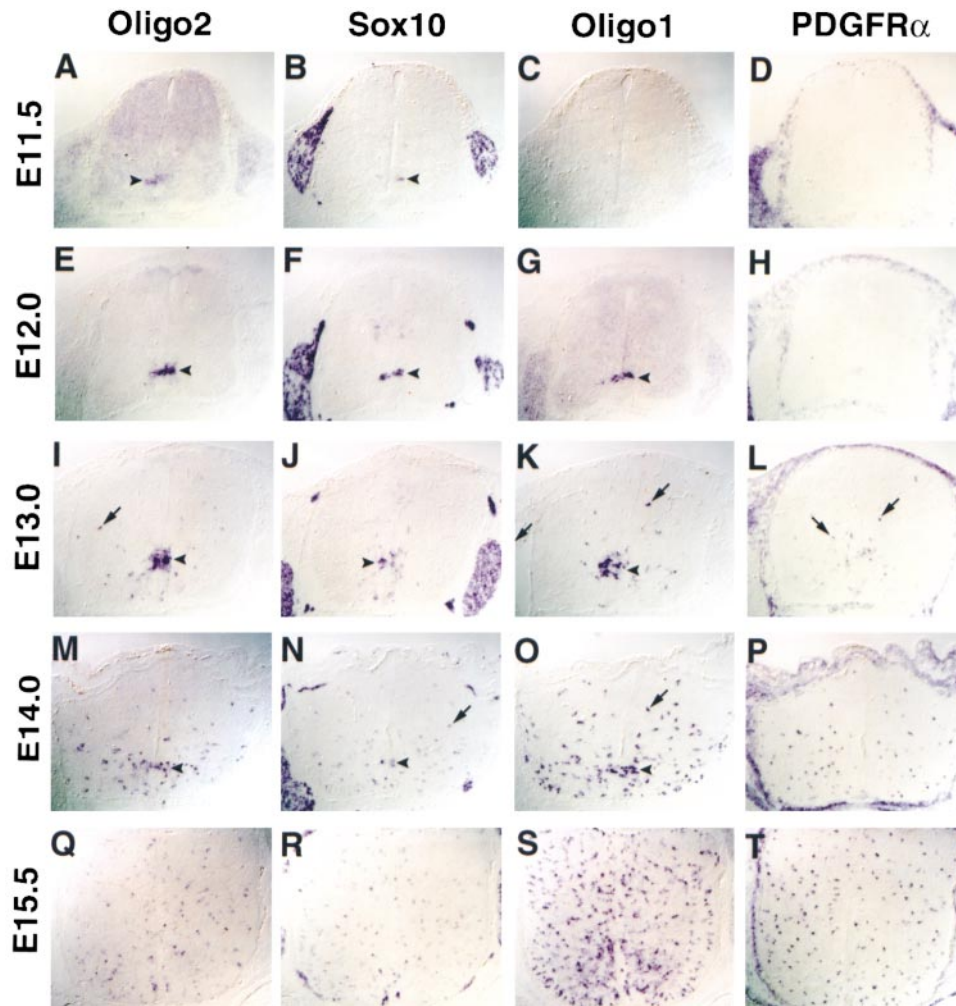


Figure 2. Expression of *Oligo1* and *Oligo2* in the Developing Spinal Cord in Relation to that of Other Oligodendrocyte Precursor Markers

Adjacent or near adjacent sections through the midthoracic spinal cord of mouse embryos at E11.5 (A–D), E12.0 (E–H), E13.0 (I–L), E14.0 (M–P), or E15.5 (Q–T) were hybridized in situ with antisense RNA probes for *Oligo1* (C, G, K, O, and S), *Oligo2* (A, E, I, M, and Q), *Sox10* (B, F, J, N, and R), and *PDGFRα* (D, H, L, P, and T). Expression of *Oligo2* precedes that of *Oligo1* and is first detected at E11.5 ([A], arrowhead). *Oligo1* is first detected at E12.0 ([G], arrowhead). *Sox10* expression is faint at E11.5 ([B], arrowhead) but is more prominent at E12.0 ([F], arrowhead). Note that expression of all three transcription factor markers at E12.0 occurs in a narrow region of the ventricular zone and precedes that of *PDGFRα* (E–H and L). Beginning at E13.0, individual cells expressing *Oligo1* or *Oligo2* can be detected in the gray matter ([I and K], arrows), similar to what is observed for *PDGFRα* ([L], arrows). By E15.5, none of the markers are detectably expressed in the ventricular zone, and all are found in scattered cells throughout the spinal cord (Q–T).

and 20, arrowheads), however, whereas *PDGFRα*⁺ cells were largely dispersed, and little residual expression in the ventricular zone was detected (Figure 2P). By E15.5, *Oligo1*⁺ and *Oligo2*⁺ cells were dispersed throughout the gray matter, and little or no residual expression in the ventricular zone was detected (Figures 2Q and 2S), a pattern like that of *PDGFRα*⁺ cells (Figures 2P and 2T). Although the number of dispersed *Oligo2*⁺ cells was similar to that of the *PDGFRα*⁺ cells, the number of *Oligo1*⁺ cells appeared greater than that of *PDGFRα*⁺ cells on adjacent sections (Figures 2S and 2T). This difference may reflect simply a difference in the sensitivity of our in situ hybridization procedure for detection of these two mRNAs; *Oligo1* signal was strong after an overnight development, while that for *PDGFRα* typically took 2 days to develop. Alternatively, a subset of migratory cells may express *Oligo1* but not *PDGFRα*.

We also compared the expression of *Oligo2* and *Oligo1* to that of another early transcriptional marker of glial cells, *Sox10*. *Sox10* encodes an HMG-box transcription factor that is expressed in neural crest cells and their derivative PNS glia, as well as in CNS astrocyte and oligodendrocyte precursors (Kuhlbrodt et al., 1998). Analysis of *Sox10* expression indicated that it was first detected at about the same time as was expression of *Oligo2* and that it preceded the expression of *Oligo1* by about 12 hr and of *PDGFRα* by about 36 hr (Figures 2B, 2F, and 2J). Double-labeling experiments indicated that *Oligo* genes and *Sox10* are likely coexpressed in the same cells (Figures 3B, 3E, and 3H; data not shown).

Taken together, these data suggest that *Oligo2* and *Oligo1* are specifically expressed by progenitors of oligodendrocyte precursors in the spinal cord in a sequential and overlapping manner (see also Figure 3G, arrows).

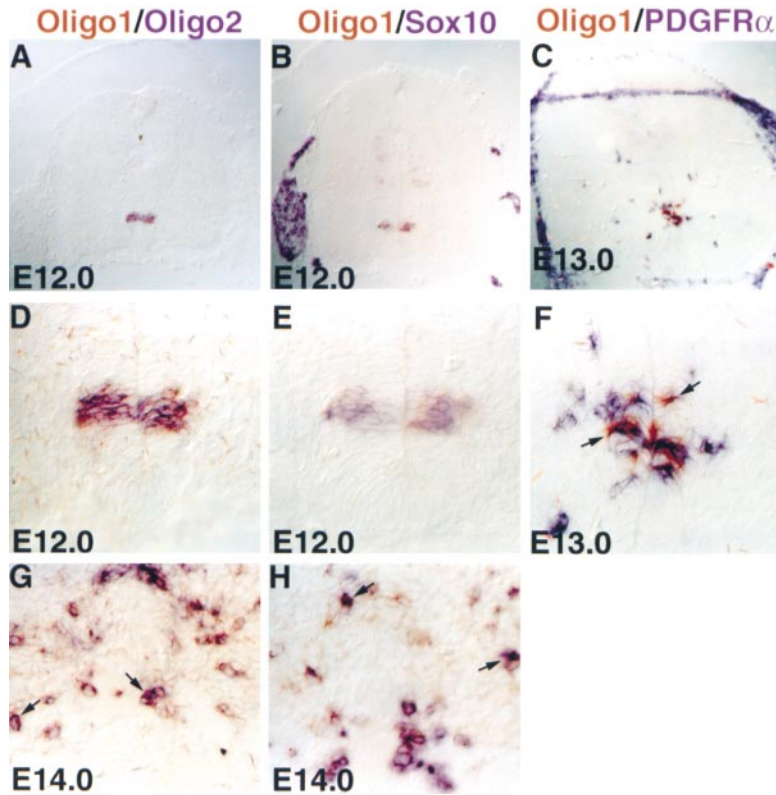


Figure 3. Coexpression of *Oligo1* with *Oligo2*, *Sox10*, and *PDGFRα*

Double-label in situ hybridization was performed on sections through midthoracic spinal cord at the indicated ages with the indicated probes. The color of the lettering of the probes corresponds to the color of the in situ hybridization reaction product. (A) and (B), and (D) and (E), represent low (10× objective) and high (40× objective) magnification views of the same fields, respectively. Note that the domains of the ventricular zone marked by expression of *Oligo1*, *Oligo2*, and *Sox10* are completely overlapping (D and E). Coexpression of these markers persists at E14.0 in migrating cells dispersed through the gray matter (G and H, arrows). At E13.0, individual cells coexpressing *PDGFRα* and *Oligo1* (arrows) can be seen to emerge from a common region of the ventricular zone (C and F).

Expression of both *Oligo* genes continues in *PDGFRα*⁺ and *Sox10*⁺ precursors as they migrate away from the ventricular zone (Figures 3H and 3F, arrows; data not shown), but *Oligo2* appears to be downregulated, while expression of *Oligo1* persists (Figures 2Q and 2S). The overlap of *Oligo* gene expression with that of *PDGFRα* and *Sox10* in both the spinal cord and in presumptive migrating oligodendrocyte precursors makes it highly unlikely that these bHLH genes are expressed in nonoligodendrocyte lineage cells coincidentally located in the same region of the ventricular zone as that from which oligodendrocytes arise. This conclusion is supported by in situ hybridization experiments in dissociated primary cultures of P5 optic nerve, which revealed *Oligo1* and *Oligo2* expression in elongated, bipolar cells with the characteristic morphology of oligodendrocyte precursors (also known as O-2A cells (Raff, 1989) (Figures 4A and 4C, arrowheads), as well as in newly formed oligodendrocytes (Figures 4B and 4D, arrows) but not in astrocytes or meningeal cells (Figures 4B and 4C, asterisks).

Expression of *Oligo* Genes in Other Regions of the CNS

We also performed a preliminary analysis of *Oligo* gene expression in other regions of the developing CNS besides the spinal cord. Foci of *Oligo* gene-expressing cells were detected in the ventricular zone of the mid-brain and hindbrain (Figures 5A–5F, arrowheads), in regions previously described as sites of oligodendrocyte precursor formation (Hardy and Friedrich, 1996). With time, *Oligo1*- and *Oligo2*-expressing cells became dispersed throughout the gray matter (Figures 5G–5L,

arrows), in a manner similar to the dispersion of oligodendrocyte precursors visualized using other markers (Hardy and Friedrich, 1996).

These data are generally consistent with the idea that *Oligo* genes are expressed in oligodendrocyte-generative zones of the neuroepithelium and in oligodendrocyte precursors in other regions of the CNS. However, low levels of *Oligo2* gene expression were also detected in the E13.5 and E14.5 retina, and both genes were expressed in the olfactory epithelium from E11.5 onward (data not shown). These data suggest that while the expression of both *Oligo* genes marks sites of oligodendrocyte precursor formation in the spinal cord and brain, these genes may also be expressed in developing peripheral sensory organs, where oligodendrocytes are not produced. At no time did we detect expression of *Oligo* genes in developing Schwann cells or their precursors, the myelinating glial cells of the PNS.

Ectopic Expression of *Oligo* Genes Causes Ectopic Expression of *Sox10* In Vivo

The timing and location of *Oligo* gene expression, taken together with their homology to other bHLH cell-type determination factors, suggested that these genes might play a causative role in some aspect of oligodendrocyte lineage determination and/or differentiation. In myogenic and neurogenic lineages, forced expression of bHLH factors has sometimes promoted expression of lineage-specific markers in heterologous cells or tissues (Davis et al., 1987; Lee et al., 1995; Ma et al., 1996). We therefore sought to carry out similar gain-of-function studies for *Oligo* genes. Efforts to detect precocious or

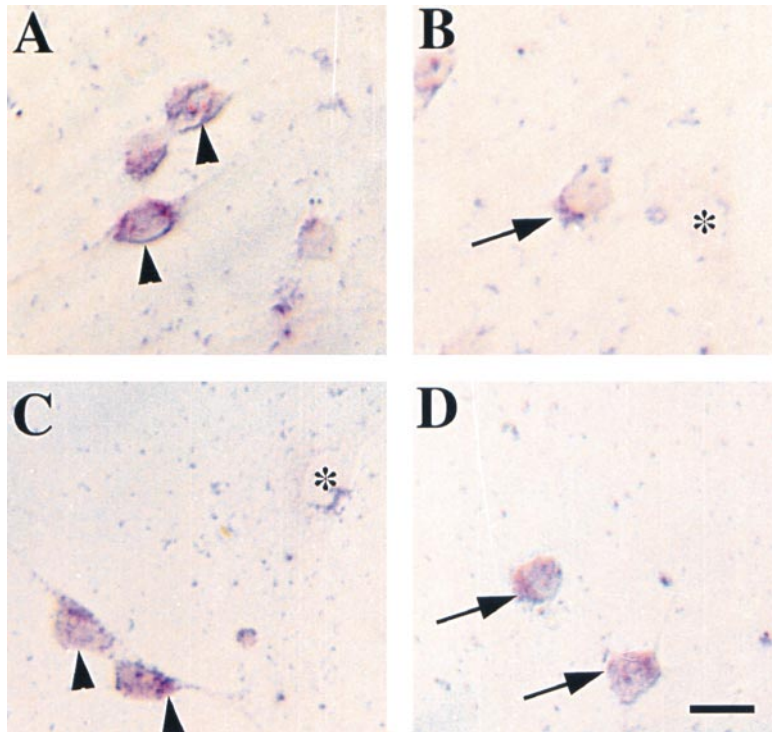


Figure 4. Expression of *Oligo1* and *Oligo2* by Primary Oligodendrocyte Precursors and Immature Oligodendrocytes

In situ hybridization to primary cultures of P5 rat optic nerve was performed using anti-sense probes for *Oligo1* (A and B) and *Oligo2* (C and D). Expression of *Oligo1* and *Oligo2* was observed in bipolar oligodendrocyte precursors ([A and C], arrowheads) and in newly formed oligodendrocytes ([B and D], arrows), which are distinguishable by their morphology from precursors. Neither gene was expressed by type 1 astrocytes ([B and C], asterisks) or meningeal cells (data not shown). Control cultures hybridized with a mixture of sense-strand *Oligo1* + *Oligo2* probes yielded no specific signal (data not shown). Scale bar, 10 μ m.

expanded expression of oligodendrocyte or oligodendrocyte precursor markers (A2B5 and O4) in cultures of rodent CNS stem cells (Johe et al., 1996) infected with retroviral vectors or transfected with expression plasmids encoding either *Oligo2* or *Oligo1* were unsuccessful under a variety of culture conditions permissive for oligodendrocyte development (Q. Z. and D. J. A., unpublished data). These data raised the possibility that environmental factors present in vivo but not in vitro might be necessary to observe the phenotypic consequences of *Oligo* expression. We therefore sought an in vivo assay of ectopic *Oligo* gene expression.

Chick embryos have proven to be a useful system for misexpression of regulatory genes in the developing nervous system because retroviruses encoding the gene of interest can be conveniently injected into the lumen of the neural tube, where they infect proliferating neural progenitors in the ventricular zone (Fekete and Cepko, 1993). In the chick, oligodendrocyte precursors emerge from a restricted zone of the ventral neuroepithelium, as they do in rodents, beginning on E6.0 (Hamburger-Hamilton stages [HH] 28–29) (Ono et al., 1995; Timsit et al., 1995; Orentas et al., 1999). Oligodendrocyte precursors can be detected using O4, an antibody that detects sulfatide and other glycolipids expressed on the surface of immature and mature oligodendrocytes (Sommer and Schachner, 1981; Ono et al., 1995). In preliminary experiments, we failed to obtain evidence of either premature or expanded expression of O4 in the spinal cords of embryos infected with *Oligo2*- or *Oligo1*-encoding retroviruses at E2.0 (HH 10–11), despite abundant expression of the retrovirally encoded transgenes (data not shown).

We therefore employed a more extensive battery of

oligodendrocyte markers in an effort to observe a phenotypic effect of *Oligo* gene misexpression. These markers included *Sox10*, *PDGFR α* , *PLP* (proteolipid protein), *DM20*, *MBP* (myelin basic protein), and the peripheral glial markers P₀ and SMP (Schwann myelin protein) (Dupin et al., 1990; Bhattacharyya et al., 1991). None of these markers yielded evidence of ectopic oligodendrocyte precursor formation or differentiation within the neural tube (data not shown). However, in embryos infected with *Oligo2*-expressing virus we noted that at E7.0, when secondary infection by the replication-competent virus had spread outside of the neural tube, apparently ectopic expression of *Sox10* could be observed in the dorsal myotome (data not shown). These data suggested that ectopic expression of *Oligo* genes might have phenotypic consequences in cells of a mesodermal lineage.

To confirm and extend these observations, retroviruses encoding *Oligo* genes (singly or in combination) were directly injected into the lumen of the somites at the epithelial ball stage (HH 10–11) to deliberately infect paraxial mesodermal precursors. Following such infections, abundant ectopic expression of *Sox10* could be detected in both the myotome and in the limb bud muscle mass of *Oligo2*-infected embryos (Figures 6C and 6D). None of the other markers employed were detected in these ectopic locations, although in some experiments there appeared to be a slightly elevated level of O4 expression in this region (data not shown). Such ectopic expression of *Sox10* was not observed with either *Oligo1*-expressing virus (Figures 6A and 6B) or a control virus expressing a nuclear localized *Luciferase* gene (Figures 6G and 6H) (Chen et al., 1998). Coinjection of *Oligo1* and *Oligo2*-expressing viruses in RCAS(A) and RCAS(B) backbones was as effective at inducing ectopic *Sox10*

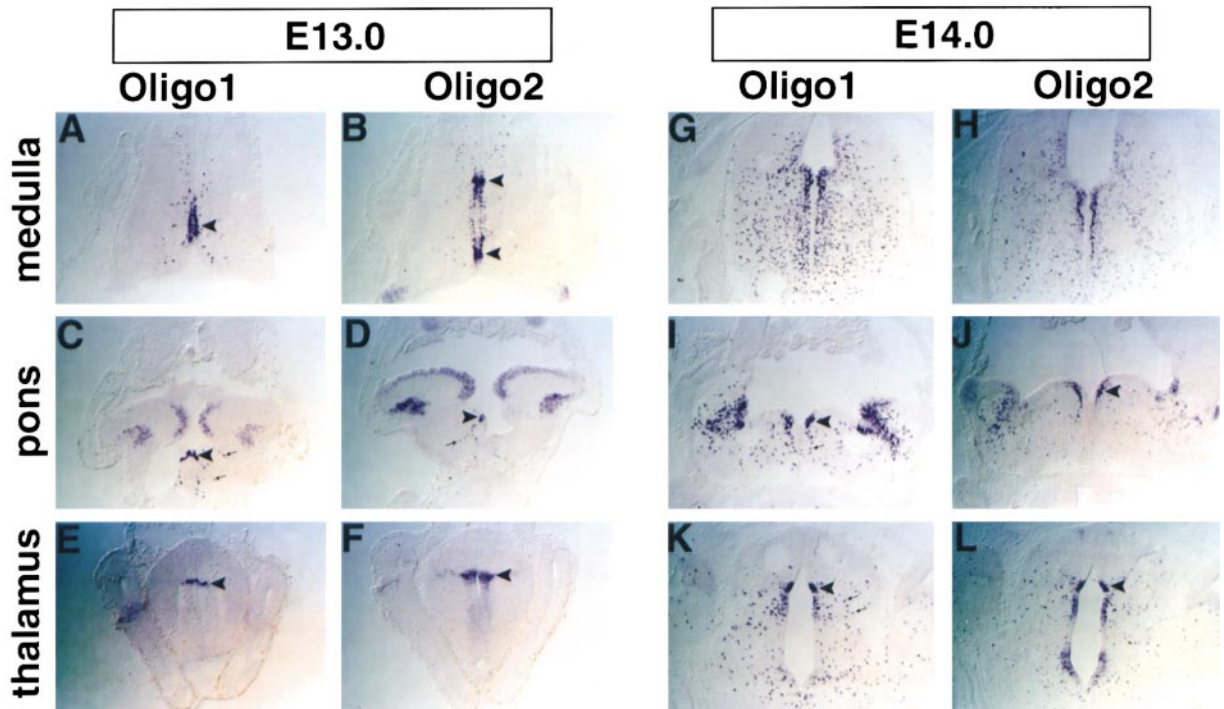


Figure 5. Expression of *Oligo* Genes in the Mid- and Hindbrain

Adjacent or near adjacent sections through the region of the medulla (A, B, G, and H), pons (C, D, I, and J), or thalamus (E, F, K, and L) at E13.0 (A–F) or E14.0 (G–L) were hybridized with *Oligo1* or *Oligo2* antisense RNA probes. Arrowheads indicate foci of *Oligo1* or *Oligo2* mRNA expression in the ventricular zone. Note that at E14.0, cells expressing *Oligo1* and *Oligo2* have begun to disperse through the gray matter (e.g., [K], arrows).

as was *Oligo2* virus alone (Figures 6E and 6F), indicating that the lack of effect of *Oligo1* on its own was not due to an inhibitory effect on *Sox10* expression.

These data suggest that ectopic expression of *Oligo2* in mesodermal lineage cells can induce *Sox10*, another early transcription factor marker for oligodendrocyte precursors, but is insufficient to promote expression of later markers. Several pieces of evidence argue that it is unlikely that the ectopic expression of *Sox10* simply reflects proliferative expansion of neural crest-derived *Sox10*⁺ Schwann cell precursors associated with peripheral nerves that course through the muscle (Figures 6B and 6H, arrowheads) rather than induction in mesodermal cells. First, the cells expressing ectopic *Sox10* in *Oligo2*-infected limbs (Figures 7C and 7E, arrowheads, purple precipitate) were distinct from peripheral nerve-associated Schwann cell precursors identified by an independent marker, HNK-1 (Bronner-Fraser, 1986) (Figures 7C and 7E, arrows, brown precipitate; see also Figures 8A, 8C, and 8E, arrows versus arrowheads). Second, the *Sox10*⁺ cells in *Oligo2*-infected limbs were distributed in the same regions as cells on near adjacent sections marked by Pax7, a transcription factor specifically expressed by myogenic precursors in the limb (Kawakami et al., 1997) (Figures 8A and 8C, arrows).

Ectopic expression of *Oligo2* in myogenic precursors did not prevent the differentiation of myogenic lineage cells, as shown not only by undiminished expression of the myoblast marker Pax7 (Figures 8A–8D) but also of the muscle differentiation marker MF-20 (Figures 8G and

8H, arrows). Although in some cases it appeared that some MF-20⁺ myocytes coexpressed *Sox10*, it was difficult to unambiguously discriminate this from labeling in overlying, closely associated *Sox10*⁺ cells, which are likely to be myoblasts (Figure 8G, arrowheads; for technical reasons, it was not possible to perform in situ hybridization for *Sox10* mRNA with antibody staining for Pax7). Taken together, these observations suggest that ectopic expression of *Oligo2* induces ectopic expression of *Sox10* in myoblasts but that this expression is largely extinguished upon differentiation of these cells to myocytes. The lack of ectopic *Sox10* expression in other tissues expressing ectopic *Oligo2*, such as skin (Figure 6, arrows), may reflect the fact that these are sites of secondary infection and therefore may have had insufficient time to respond by the time of fixation. Alternatively, mesoderm-derived precursors may be especially permissive for activation of *Sox10* by *Oligo2*.

Discussion

bHLH transcription factors control the development of a number of differentiated cell types, including different classes of neurons (Kageyama and Nakanishi, 1997; Lee, 1997), but the members of this family involved in glial development have not previously been described. Here, we identify two closely related bHLH factors, called *Oligo2* and *Oligo1*, that are specifically expressed in both oligodendrocyte precursors and in the zone of the neuroepithelium from which they arise. These genes

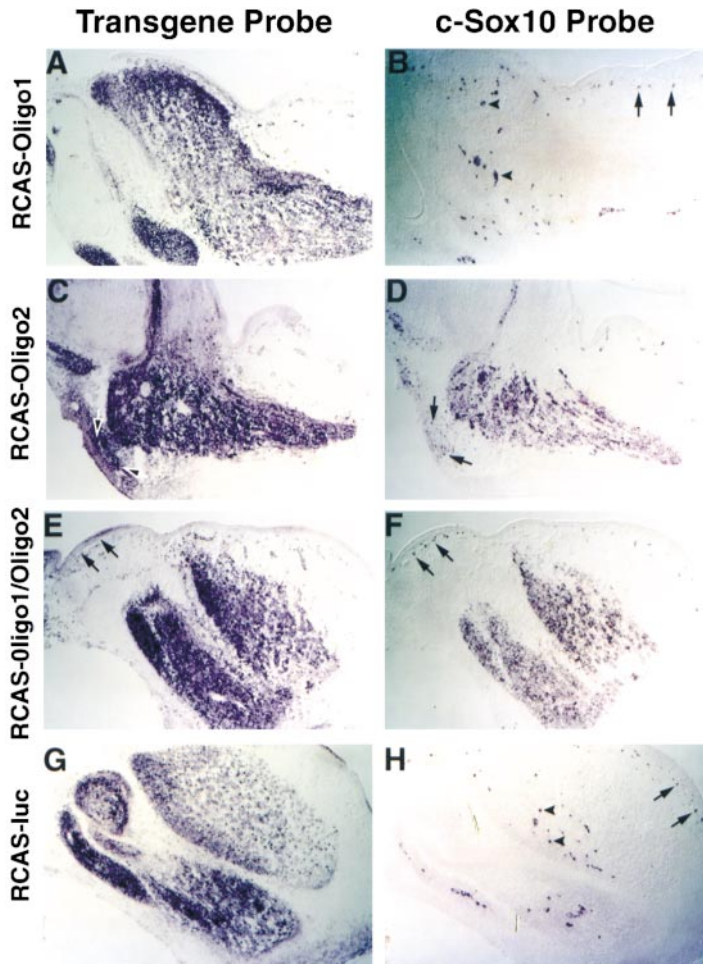


Figure 6. Ectopic Expression of *Oligo2* in Somite-Derived Mesodermal Cells Induces Ectopic *Sox10* Expression in Chick Embryos In Vivo

Sections through the limb muscle mass of embryos infected with replication-competent retroviruses at E2.0 and harvested at E7.0 are shown.

(A, C, E, and G) The sections were hybridized with a probe corresponding to the transgene expressed by the viruses indicated to the left, to visualize the extent of infection/expression. Note that in (E), only *Oligo2* hybridization is shown, although the region illustrated also expressed *Oligo1* (data not shown).

(B, D, F, and H) Sections adjacent to those shown in (A), (C), (E), and (G), respectively, were hybridized with a *Sox10* antisense RNA probe. Note that RCAS-Oligo2 or RCAS-Oligo1 + RCAS-Oligo2 caused extensive ectopic expression of *Sox10* in the muscle mass (D and F). No such ectopic expression was observed with RCAS-Oligo1 (B) or RCAS-luciferase viruses (H). Scattered *Sox10*-expressing cells in (B) and (H) are neural crest derived cells in the subectodermal layer (arrows) or Schwann cell precursors associated with peripheral nerve fibers in the muscle (arrowheads). As shown in Figures 6 and 7, the ectopic expression of *Sox10* does not result from proliferative expansion of these *Sox10*⁺ nerve-associated cells. Note that expanded expression of *Sox10* is not seen in well-infected areas of skin, where individual *Sox10*⁺ cells are visible [(C and D), arrows].

are expressed substantially earlier than is *PDGFR α* , the earliest marker of the oligodendrocyte lineage described previously (Pringle and Richardson, 1993), and prospectively identify the region of the neural tube that will generate oligodendrocytes over 1 day before this process begins. Ectopic expression of *Oligo2* in vivo induces ectopic expression of *Sox10*, a glial lineage-specific HMG-box transcription factor (Kuhlbrodt et al., 1998), in mesodermal lineage cells. Together, these expression and gain-of-function data suggest that *Oligo* genes are likely to play an important role in early stages of oligodendrocyte lineage determination.

Oligo Genes May Be Involved in a Switch from the Production of Neurons to Oligodendrocytes in the Spinal Cord

Oligodendrocytes arise from the same region of the spinal cord that generates motor neurons (Sun et al., 1998) but differentiate after the production of these neurons is mostly completed. The observation that oligodendrocyte precursors can share a common progenitor with neurons (Leber et al., 1990; Williams et al., 1991) has been taken to imply that ventricular zone cells in the spinal cord must switch from producing neurons to oligodendrocytes (Sun et al., 1998; Orentas et al., 1999). However, apparently glial restricted precursors have recently been isolated from the embryonic rat spinal cord, which generates only astrocytes or oligodendrocytes

under culture conditions that are permissive for neuronal differentiation (Rao et al., 1998; Mujtaba et al., 1999). Whether the induction of *Oligo2* occurs in a progenitor common to neurons and oligodendrocytes or in cells that are already restricted to a glial fate remains to be determined. Whatever the case, the early expression of *Oligo2* suggests that the initial events in the specification of the oligodendrocyte lineage may have already occurred at least 1 day before oligodendrocyte precursors can be visualized by expression of *PDGFR α* .

Whatever their lineage relationship to motor neurons, oligodendrocyte precursors are induced in a specific zone of the neuroepithelium at a specific time. The factors controlling this induction are not fully identified. Shh is known to be both necessary and sufficient for oligodendrocyte as well as motor neuron differentiation (Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996; Tanabe and Jessell, 1996). Antibody blocking studies have shown that Shh is required immediately before the production of oligodendrocyte precursors, when many motor neurons have already differentiated (Orentas et al., 1999). This late requirement for Shh could reflect a role in the induction of *Oligo* gene expression. Consistent with this idea, misexpression of Shh dorsally induces ectopic *Oligo* gene expression in the dorsal neural tube (Lu et al., 2000). However, it is currently not clear whether this is an indirect consequence of the effect of Shh to ventralize the neural tube (Ericson et al.,

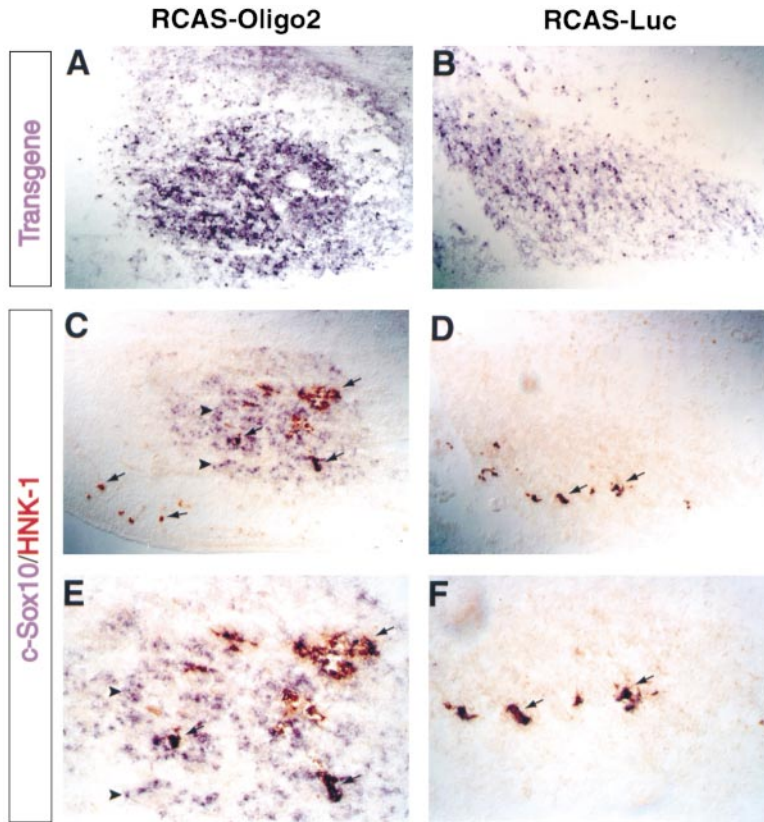


Figure 7. Ectopic Sox10⁺ Cells in *Oligo2*-Infected Limb Are Distinct from Peripheral Nerve-Associated Neural Crest-Derived Schwann Cells

Limbs of E7.0 embryos injected at E2.0 with either RCAS-*Oligo2* (A, C, and E) or RCAS-luciferase (B, D, and F) viruses were analyzed for expression of the retrovirally encoded transgenes (A and B) or double labeled (C–F) for *Sox10* mRNA (purple) and HNK-1 antibody staining (brown). (A) and (C), and (B) and (D), are near adjacent sections; (E) and (F) are higher magnification views of the fields shown in (C) and (D), respectively. Neural crest-derived Schwann cells associated with peripheral nerve fibers in the muscle express both *Sox10* and the HNK-1 epitope [(C–F), arrows]. However the cells expressing ectopic *Sox10* in the muscle mass [(C and E), arrowheads] are only purple (i.e., HNK-1⁻) and therefore are not Schwann cells.

1996) or a more direct effect on *Oligo* gene expression. Whatever the case, the fact that *Shh* is required for the sequential differentiation of both the neuronal and glial lineages indicates that it is unlikely to explain the timing of oligodendrocyte production. Identification of the factors controlling the temporal onset of *Oligo* gene expression may help to shed light on this issue.

Expression of *Oligo2* appears restricted to the ventral spinal cord from the earliest stages we have examined (E9.5) but is initially expressed in a much broader zone than it is at later stages (E11.5). A similar narrowing of the zone of *Oligo1* expression is seen as well, although this gene is expressed at much lower levels than *Oligo2* is at such early stages. The significance of this early expression of *Oligo* genes, particularly that of *Oligo2*, is not clear. Although it occurs around the time when motor neurons are being generated, we did not observe any evidence of ectopic neuronal marker expression in either the spinal cord or the paraxial mesoderm of embryos ectopically expressing *Oligo2* (data not shown). One possibility is that the early phase of *Oligo2* expression controls the timing of motor neuron generation by temporarily inhibiting such differentiation in the zone where it is expressed. Alternatively, *Oligo2* may play a positive acting role in the differentiation of motor neurons that has thus far escaped detection in our functional assays.

Ectopic Expression of *Oligo* Genes Promotes Ectopic Expression of *Sox10*

Sox10 has been shown to be expressed in glial lineages in both the CNS and PNS (Kuhlbrodt et al., 1998), but the precise temporal relationship of its expression to that of other oligodendrocyte markers has not previously

been investigated in detail. Here, we show that *Sox10* is abundantly expressed in the spinal cord at sites of oligodendrocyte production and is induced at about the same time as *Oligo2* and before both *Oligo1* and *PDGFR α* . Expression of *Sox10* also persists in migratory oligodendrocyte precursors that express the other genes. A dominant mutation in murine *Sox10*, called *Dom*, produces pigmentation defects and agangliogenesis of the bowel in heterozygotes (Southard-Smith et al., 1998), reflecting its early expression in neural crest cells. The embryonic lethality obtained in *Dom/Dom* homozygotes precludes an assessment of its role in myelination, which occurs postnatally. That *Sox10* is functionally important for oligodendrocyte development, however, is suggested by the recent discovery of a human mutation in the gene that is associated with a CNS dysmyelinating disease (Inoue et al., 1999). The fact that ectopically expressed *Oligo2* is able to promote ectopic expression of *Sox10* in vivo therefore provides evidence of a functional role for *Oligo2* in oligodendrocyte lineage determination.

The inability of *Oligo1* to induce ectopic *Sox10* expression is correlated with the fact that it is normally expressed after *Sox10* and *Oligo2*. The sequential expression of structurally related bHLH transcription factors is a characteristic feature of many differentiating lineages (Jan and Jan, 1993); for example, muscle precursors sequentially express first *MyoD* (or *Myf5*) and then *Myogenin* (Molkentin and Olson, 1996), while precursors of peripheral sensory neurons first express one of the *Neurogenins*, followed by one or more members of the *NeuroD* subfamily (Anderson, 1999). Such sequential expression of bHLH genes is thought to underly the linked

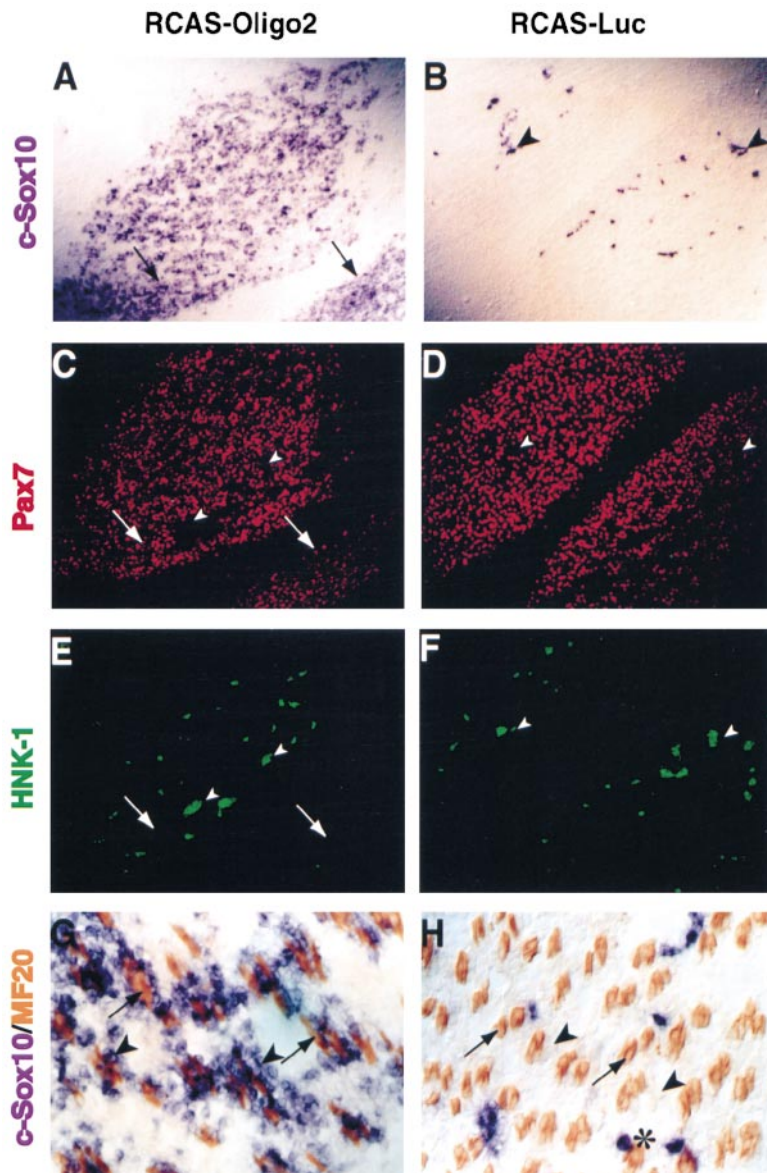


Figure 8. Ectopic *Sox10* Expression in the Limb Is Compatible with Muscle Differentiation

Sections of limb from embryos injected with either RCAS-*Oligo2* (A, C, E, G, and H) or RCAS-luciferase (B, D, and F) were processed for in situ hybridization with a chick *Sox10* probe (A and B), double labeled with antibodies to Pax7 (C and D) and HNK-1 (E and F), or hybridized for *Sox10* expression and counterstained with antibody MF-20, a muscle-specific marker (G and H). (C) and (E), and (D) and (F), are images of the same field viewed with different epifluorescence filters and are taken from sections near to those shown in (A) and (B), respectively. Note that ectopic *Sox10* expression is induced in regions of the muscle ([A], arrows) that contain Pax7⁺ myoblasts ([C], arrows) but not HNK-1⁺ Schwann cell precursors ([E], arrowheads; see also [B], [D], and [E], arrowheads). Clusters of ectopic *Sox10*⁺ cells in *Oligo2*-infected muscle ([G], arrowheads) are closely associated with MF-20⁺ myocytes ([G], arrows), some of which may also express *Sox10*. The position and morphology of the *Sox10*⁺ cells are similar to those of *Sox10*⁻ presumptive myoblasts ([H], arrowheads), which are associated with MF-20⁺ myocytes in the contralateral, uninfected limb of an *Oligo2*-infected embryo ([H], arrows). Asterisk indicates *Sox10*⁺ Schwann cell precursors associated with peripheral nerve fiber (see also [F], arrowheads).

processes of determination and differentiation (Weintraub, 1993). Interestingly, studies of the myogenic bHLH family suggest that the earlier acting determination genes, *MyoD* and *Myf5*, are more efficient at inducing muscle markers in heterologous cells than is the downstream differentiation gene *Myogenin* and that this difference may be due to a differential ability to alter chromatin structure (Gerber et al., 1997). Such a difference might likewise account for the differential ability of *Oligo2* and *Oligo1* to activate *Sox10* in mesodermal lineage cells. The fact that *Oligo1* is highly homologous to *Oligo2* in the bHLH domain and is expressed in the same cells as *Oligo2* and *Sox10* suggests that it may play a role in the maintenance rather than the initiation of *Sox10* expression.

None of the six other markers of oligodendrocytes or glia that we examined (O4, PLP, DM20, MBP, SMP, and P₀) were convincingly ectopically activated by *Oligo2* in mesodermal cells (although a weak elevation of O4

expression over background was sometimes apparent). That forced expression of *Oligo2* fails to recapitulate the complete process of oligodendrocyte differentiation, particularly in cells of a heterologous lineage, is not entirely unexpected. Although *MyoD* and other muscle-specific bHLH factors can induce full myogenic differentiation when ectopically expressed in some heterologous cells (Davis et al., 1987), this activity is not observed in all cell types (Weintraub et al., 1989), nor is it characteristic of all other tissue-specific bHLH factors that have been studied (see, for example, Johnson et al., 1992). The ability of *Oligo2* to activate *Sox10* but not other markers is consistent with the fact that *Sox10* is the earliest of these markers to be expressed and suggests that *Sox10* may be a direct target of *Oligo2*. The failure of *Oligo2* to activate later oligodendrocyte markers may reflect its inability to override the myogenic determination program of mesodermal cells. In support of this idea, forced expression of the *Oligo* genes did

not prevent activation of the normal myogenic program, as detected by expression of both the myoblast marker Pax7 and the muscle differentiation marker MF-20.

It may seem paradoxical that ectopic expression of *Oligo2* activated ectopic *Sox10* expression in mesodermal lineage cells but not in neural lineage cells, where it normally acts. However, the inability of ectopic *Oligo* gene expression to override the endogenous program of neurogenesis in other regions of the neuroepithelium may reflect the tight spatial restriction on the generation of oligodendrocytes in the spinal cord. Perhaps this localization involves not only the restricted expression of presumptive positive regulators of the oligodendrocyte fate, such as the *Oligo* genes, but also active inhibition of oligodendrocyte lineage determination in other regions. If so, then the action of ectopically expressed *Oligo* genes may be prevented by such inhibitors. As the mesoderm normally produces neither neurons nor oligodendrocytes, there would be no reason to evolve such an inhibitory mechanism, explaining the ability of ectopic *Oligo2* expression to activate *Sox10* in this non-neural tissue. The mechanism of such inhibition will be an interesting topic for further exploration.

Experimental Procedures

Isolation and Characterization of Murine *Oligo1* and *Oligo2* Genes

To identify novel bHLH transcription factors, a BLAST search of the GenBank database was performed with the bHLH domain of different families of known bHLH transcription factors. Two unknown bHLH factors were identified in this process, residing on a BAC clone (GenBank accession number AP000041) from human chromosome 21q. Based on these human sequences, degenerate primers were designed and used to PCR amplify and clone partial sequences of the mouse homologs of these genes. The template used in the PCR amplification was mouse genomic DNA. After preliminary in situ data suggested that these two genes had an interesting expression pattern in the nervous system, full-length genomic sequences were obtained through screening a mouse genomic library (129SvJ, λ phage library, Stratagene).

In Situ Hybridization

Nonradioactive in situ hybridization on frozen sections of mouse and chick embryos was performed as previously described (Ma et al., 1996; Perez et al., 1999). The following probes were used: mouse *Oligo1*, mouse *Oligo2*, rat *Sox10* (a gift of Dr. Kirsten Kuhlbrodt), mouse *PDGFR α* , chick *Sox10*, chick *MBP*, and chick *PLP/DM20*.

Double in situ hybridization was performed as previously described (White and Anderson, 1999). The two different probes were labeled with either digoxigenin- or fluorescein-UTP. The first probe was detected with alkaline phosphatase- (AP-) conjugated anti-digoxigenin antibody and developed with Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), which yields a purple precipitate. After the NBT/BCIP reaction, the AP-conjugated anti-digoxigenin antibody was inactivated at 85°C; the slides were subsequently incubated with AP-conjugated anti-fluorescein antibody and detected with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride and BCIP, which yields a reddish brown precipitate.

In situ hybridization to primary cultures of P5 rat optic nerve was performed after 2 days of growth in serum-free medium containing PDGF (Barres et al., 1992, 1993). Hybridization was performed with 2 μ g/ml fluorescein-UTP-labeled probe overnight at 58°C. Cultures were developed by overnight incubation with anti-fluorescein antibody diluted 1:1000 at 4°C, and the AP reaction was developed for 36 hr at room temperature.

Immunohistochemistry

Mouse embryos were fixed by immersion in 4% paraformaldehyde overnight at 4°C. Chick embryos were fixed for 2–3 hr at 4°C. The

following primary antibodies were used at the indicated dilutions: O4 (1:2, hybridoma, ATCC), P₀ (1:10, hybridoma, ATCC), anti-SMP (undiluted, hybridoma, Developmental Studies Hybridoma Bank), MF-20 (1:10, hybridoma, Developmental Studies Hybridoma Bank), and Pax7 (1:1000, ascites, Developmental Studies Hybridoma Bank). Secondary antibodies used were goat-anti-mouse-immunoglobulin M-FITC (Jackson), goat-anti-mouse-immunoglobulin G-horse-radish peroxidase (Chemicon), and goat-anti-mouse-immunoglobulin G-Alexa 568 (Molecular Probes).

Immunohistochemistry after in situ hybridization was performed essentially as described above with the following modification. To better preserve protein antigens, proteinase K digestion in the in situ procedure was lowered from 20 μ g/ml for 5 min to 10 μ g/ml for 2 min. Detailed protocols are available upon request.

Retroviral Infection Experiments

The murine *Oligo1* gene with one Myc epitope tag at the amino terminus was cloned into RCASBP(A) (Morgan and Fekete, 1996), and the murine *Oligo2* gene with one Myc tag at the amino terminus was cloned into the RCASBP(B) (Morgan and Fekete, 1996). The control virus used is an RCASBP(B)-based luciferase with a nuclear localization signal and 5 Myc tags at the amino terminus (Chen et al., 1998). Viral production was carried out essentially as described in Perez et al. (1999). Virus was injected unilaterally into somites at the forelimb level of animals at HH 13–15 (somites 15–19) (Christ and Ordahl, 1995), either singly or in combination. Animals were harvested at E5.0 or E7.0.

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GenBank Accession Numbers

The GenBank accession numbers for the mouse *Oligo1* and *Oligo2* sequences reported in this paper are AF232928 and AF232929, respectively.

Note Added in Proof

These authors and the authors of the related paper by Lu et al. agree that in all subsequent publications these genes shall be named *Olig1* and *Olig2*.