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Neuropathy-Associated Egr2 Mutants Disrupt Cooperative Activation of Myelin Protein Zero by Egr2 and Sox10[▽]

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Dominant mutations in the early growth response 2 (Egr2/Krox20) transactivator, a critical regulator of peripheral myelin development, have been associated with peripheral myelinopathies. These dominant mutants interfere with the expression of genes required for myelination by Schwann cells, including that for the most abundant peripheral myelin protein, *Myelin protein zero* (*Mpz*). In this study, we show that Egr2 mutants specifically affect an Egr2-responsive element within the *Mpz* first intron that also contains binding sites for the transcription factor Sox10. Furthermore, Egr2 activation through this element is impaired by mutation of the Sox10 binding sites. Using chromatin immunoprecipitation assays, we found that Egr2 and Sox10 bind to this element in myelinating sciatic nerve and that a dominant Egr2 mutant does not perturb Egr2 binding but rather attenuates binding of Sox10 to the *Mpz* intron element. Sox10 binding at other sites of Egr2/Sox10 synergy, including a novel site in the *Myelin-associated glycoprotein* (*Mag*) gene, is also reduced by the dominant Egr2 mutant. These results provide the first demonstration of binding of Egr2/Sox10 to adjacent sites in vivo and also demonstrate that neuropathy-associated Egr2 mutants antagonize binding of Sox10 at specific sites, thereby disrupting genetic control of the myelination program.

Egr2/Krox20 (hereafter referred to as Egr2) is a transactivator required for myelination of the peripheral nervous system by Schwann cells (22, 45). The induction of Egr2 at the onset of myelination coincides with the induction of major myelin genes, and analyses of Egr2-deficient mice (22, 45) and ectopic Egr2 expression in primary Schwann cells (32) have shown that Egr2 regulates myelin genes, such as the *Myelin protein zero* (*Mpz*), *Myelin-associated glycoprotein* (*Mag*), *Connexin-32* (*Cx32*/*GJB1*), and *Peripheral myelin protein 22* (*Pmp22*) genes. Egr2 is also involved in blocking proliferation as Schwann cells differentiate (35, 53).

One of the outstanding enigmas regarding Egr2 activity is the mechanism by which dominant mutants of Egr2 cause peripheral neuropathies. Dominant neuropathy-associated mutations have been identified in all three zinc fingers of the DNA-binding domain (3, 29, 34, 43, 46), and these mutations generally impair or prevent DNA binding (30, 31, 47). Previous work indicated that the dominant Egr2 mutants exert a dominant-negative effect on activation of endogenous target genes in Schwann cells by wild-type Egr2 (2, 32).

One puzzling aspect of the dominant Egr2 mutants is that their expression affects only a subset of Egr2 target genes. One of the most dramatically affected genes is the *Mpz* gene, which produces the most abundant protein (known as P_0) in peripheral myelin and is commonly mutated in human peripheral neuropathies (reviewed in references 40 and 51). However, the effect of the dominant-negative mutants was observed only in the context of Egr2 activation of endogenous *Mpz* and not with a transfected *Mpz* promoter construct (32). These data lead us to speculate that other regulatory elements of the *Mpz* gene are targeted by dominant Egr2 mutants associated with peripheral neuropathies. *Mpz* is expressed at a low level during embryonic development of Schwann cells from the neural crest and is then induced further at the onset of myelination. The Sox10 transcription factor binds to several sites in the *Mpz* promoter and is required for the embryonic expression of *Mpz* in developing Schwann cells (37). Based on transgenic experiments indicating functional elements downstream of the *Mpz* transcription start site (7), we have recently identified an element within the first intron of the *Mpz* gene (24). The following experiments describe a unique role for this element in the mechanism by which dominant-negative Egr2 mutants deregulate *Mpz* expression.

MATERIALS AND METHODS

Plasmids. Luciferase reporters containing the *Mpz* first intron element, the *Nab2* promoter, and multimerized Egr binding sites have been described previously (11, 24, 39). Mutations equivalent to neuropathy-associated mutations (R359W, S382R\D383Y, R409W) (43, 46) were introduced into an expression vector for mouse Egr2 (39). The numbering of the residues in mouse Egr2 is slightly different (356, 379/380, and 406, respectively), but the human numbering system is used for the sake of simplicity. Site-directed mutagenesis of the *Mpz* intron reporter was performed to alter the indicated Sox10 sites to G at positions 4 and 5 on the CA-rich strand, which has been previously reported to abrogate Sox10 DNA binding (4). The Sox10 expression construct (provided by Robin Miskimins) was previously described (20). The pCMVSport6-Sox11 expression vector was obtained as I.M.A.G.E. clone 5716171 (Invitrogen).

Electrophoretic mobility shift assays (EMSAs). Recombinant Egr2 and Sox10 proteins were incubated for 20 min with 5 pmol of FAM (6-carboxyfluorescein)-labeled DNA fragments amplified from the *Mpz* first intron reporter plasmids (nucleotides 1201/1320 relative to the mouse *Mpz* transcription start site) that were either wild type or mutated in the Egr2 or Sox10 binding sites. Binding reaction mixtures included a nonspecific 20-bp oligonucleotide in binding buffer (10% glycerol, 20 mM Tris [pH 7.5], 130 mM KCl, 5 mM $MgCl_2$, 0.01 mM $ZnCl_2$, 2 mM dithiothreitol, 0.1% Triton X-100) in a volume of 20 μl. Samples were electrophoresed on native 4% polyacrylamide gels and imaged using the Storm 840 system (Molecular Dynamics). Recombinant Egr2 (see Fig. 5) was made by

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fusing the mouse Egr2 sequence with the six-His tag in pET30a (Novagen) and purifying the protein from bacteria using Ni-nitrilotriacetic acid agarose (QIAGEN) according to the manufacturer's protocol. In addition, six-His-tagged Egr2, Egr2 (SR/DY), and Egr2 Δ1-180 (11), also containing an N-terminal hemagglutinin (HA) epitope, were generated by cloning them in frame with the polyhistidine tag in pCITE3a. FLAG-Sox10 protein was generated by inserting the mouse Sox10 sequence with an N-terminal 3× FLAG epitope in pcDNA3.1. These plasmids were transcribed and translated in vitro using the TNT quick system (Promega) and purified using either an anti-FLAG affinity resin (Sigma) or MagneHIS affinity beads (Promega).

Transfection assays. The S16 and S16Y rat Schwann cell lines (44) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine growth serum (HyClone). HeLa cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum. B16/F10 mouse melanocytes were obtained from ATCC. Primary rat Schwann cells were cultured as described previously (25). Transfection assays were performed as described previously (24). Induction (*n*-fold) is calculated relative to the luciferase activity of the reporter alone. Unless otherwise indicated, means and standard errors are representative of three independent experiments performed in duplicate.

Quantitative PCR analysis, Western blot analysis, and coimmunoprecipitation. S16 cells were placed in N2 medium 24 h prior to infection with adenoviruses (Ads) (1.1×10^{10} PFU/ml), prepared using the AdEasy system (13) and expressing either green fluorescent protein (GFP) or the neuropathy-associated SR/DY mutant, and then placed in growth medium for 24 h before being harvested. After 48 h, RNA was purified using the TRIzol reagent (Invitrogen), and quantitative reverse transcription-PCR was performed as described previously (25). The relative amounts of the genes were determined using the comparative threshold cycle method (26) and normalized to the relative levels of 18S rRNA. Primer sequences are available upon request.

For coimmunoprecipitation, the purified six-His HA-Egr2, HA-Egr2Δ1-180, and FLAG-Sox10 proteins were mixed and immunoprecipitated using M2 anti-FLAG-agarose beads (Sigma) according to the manufacturer's recommendations. After the final wash, proteins were eluted by boiling them for 3 min in 1× Laemmli buffer prior to immunoblot analysis using a polyclonal antibody directed against the HA epitope (Sigma).

Lysates from infected S16 cells were analyzed by immunoblotting for Sox10 using a 1:500 dilution of a polyclonal antibody (Chemicon) or using a 1:300 dilution of a polyclonal antibody for Egr2 (PRB-1567; Covance). The membranes were probed with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Jackson Laboratories, Bar Harbor, ME) at a dilution of 1:10,000. Luminescence was detected with West pico chemiluminescence reagents (Pierce, Rockford, IL) using the AutoChemi imaging system (UVP, Upland, CA).

ChIP. For in vivo chromatin immunoprecipitation (ChIP), sciatic nerves were dissected from Sprague-Dawley rat pups at postnatal day 10. ChIP assays with S16 cells and pooled sciatic nerves were performed as previously described (16), using 2 μg of anti-Krox20 antibody (Covance) and 2 μg of anti-Sox10 (ab25978; Abcam). Values are expressed as percent recovery compared to the level of input into the immunoprecipitation as determined by quantitative PCR. The primer sets used for quantitative PCR analysis of the ChIP assays were as follows: for *Mpz* −2.3 kb, 5′-AGCACCAGAGGACAAATACAC-3′ (forward) and 5′-ATGTGCTCCACTGTTTTGCCT-3′ (reverse); for the *Mpz* promoter, 5′-CTTTCTGTCCCTCTGCCTCA-3′ and 5′-TCCCCCTACCCTAGGTTGGA-3′ (reverse); for intron 1, 5′-AGAGCCAGCCCACACACATAG-3′ (forward) and 5′-GCCCACACAGGGAGAGTCAT-3′ (reverse); for intron 5, 5′-TGATCATGTCCACCCGTCCC-3′ (forward) and 5′-GGCAGGTTAGCGGGATTTG-3′ (reverse); for the *Cx32* promoter, 5′-CAGATCAAACGCCCTGACTTC-3′ (forward) and 5′-GCTGGGACACAAGTGCTCTGT-3′ (reverse); for the *IgG2a* promoter, 5′-GAAATTCTGCCCTGCACTTCC-3′ (forward) and 5′-GCTTTGCATTGAGGGAGGATC-3′ (reverse); for *Mag* intron 2, 5′-CAGGAATTCACACGGCATGC-3′ (forward) and 5′-GAGTGCAGTGAAGGAGCAGAT-3′ (reverse); and for the *Nab2* promoter, 5′-ATAGCTCGGCCTCGGTCAC-3′ (forward) and 5′-GGGACTCAAGAATCGGGCTC-3′ (reverse).

RESULTS

Egr2 activation of the *Mpz* intron element is sensitive to dominant, neuropathy-associated Egr2 mutants. Initial characterization of dominant Egr2 mutants showed that they affected endogenous *Mpz* expression but did not affect the *Mpz* promoter (32). We recently characterized a novel Egr2-responsive element within the first intron of the *Mpz* gene (24). To determine whether this element plays a role in the downregulation of *Mpz* activation by Egr2 mutants, primary rat Schwann cells were transfected with a reporter containing the *Mpz* intron element upstream of a minimal promoter. Luciferase activity was induced 17-fold over its basal activity by cotransfection of an Egr2 expression plasmid, but cotransfection of three neuropathy-associated Egr2 mutants (the S382R/D383Y [hereafter referred to as SR/DY], R359W, and R409W mutants) resulted in a dose-dependent reduction in promoter activity (Fig. 1B). Similar results were observed in three

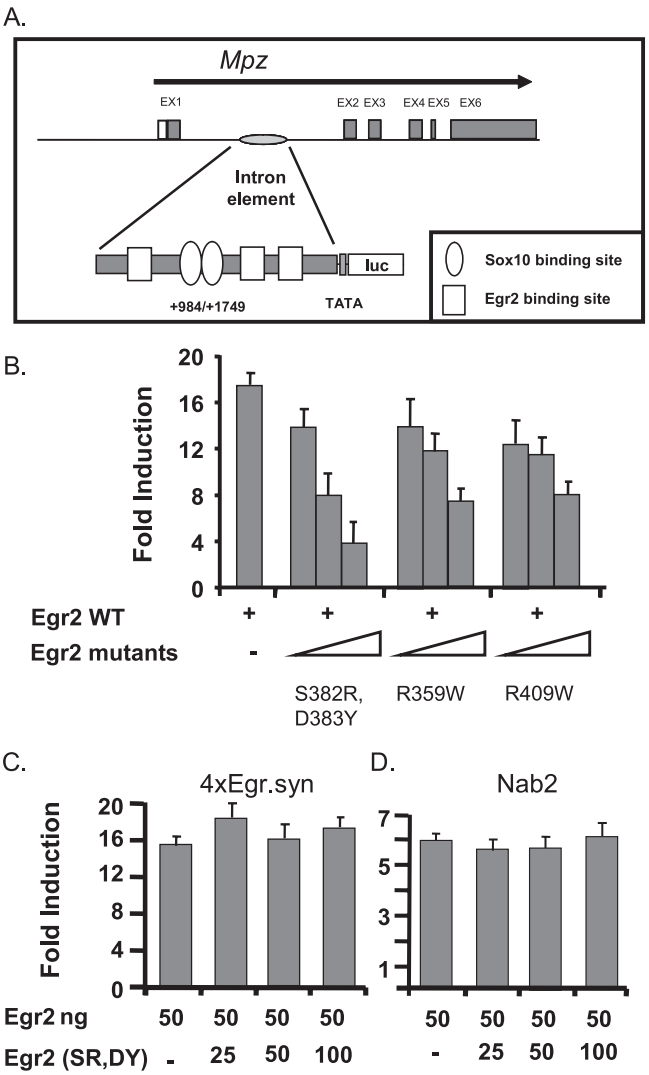

FIG. 1. Identification of a conserved region within the *Mpz* first intron that is sensitive to dominant mutants of Egr2. (A) The diagram of the *Mpz* locus indicates the position of the conserved intron element of *Mpz*. The putative Sox10 and Egr2 binding sites are indicated with ovals and squares, respectively. (B) Rat Schwann cells were cotransfected with a luciferase reporter construct containing the conserved region of the *Mpz* first intron (+984/+1749) and expression plasmids for wild-type (WT) Egr2 (50 ng) and the indicated Egr2 mutants (25, 50, and 100 ng). Similar assays were performed with luciferase reporters containing four consensus Egr binding sites upstream of a minimal promoter (4xEgr.syn) (C) or the *Nab2* promoter (D). Induction is calculated relative to the activity of the reporter alone. EX, exons of the *Mpz* gene.

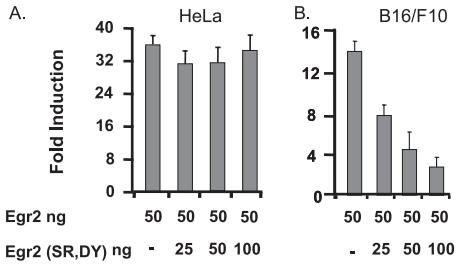

FIG. 2. Dominant-negative Egr2 mutants selectively inhibit in a cell type-specific manner. HeLa cells (A) or B16/F10 melanocytes (B) were transfected as described for Fig. 1 with the *Mpz* intron element reporter and the indicated amounts of expression plasmids for Egr2 and the dominant S382R/D383Y (SR/DY) mutant.

Schwann cell lines (MSC80, S16, and S16Y) (data not shown). This provides the first example of a specific response element that recapitulates the dominant action of the Egr2 mutants.

In order to assess whether the results were specific to the *Mpz* intron element, we repeated these studies using a luciferase reporter construct containing four consensus Egr2 binding sites (Fig. 1C) as well as the Egr2-responsive *Nab2* promoter (11) (Fig. 1D). On both reporters in Schwann cells, no dominant activity by the mutant (SR/DY) was observed, and previous experiments had shown that the *Mpz* promoter was not affected by dominant-negative Egr2 mutants (32). These results suggest that the dominant-negative effect of Egr2 mutants on *Mpz* expression is dependent on sequence elements within the conserved intron element. Furthermore, the dominant Egr2 mutants do not appear to target a factor that is required for all Egr2-dependent transactivation.

One possibility is that dominant Egr2 mutants disrupt interactions between Egr2 and another factor that binds to the *Mpz* intron element. To determine whether such a factor was Schwann cell specific, we tested the dominant-negative effect in other cell lines. Activation of the intron element by Egr2 was observed in both the HeLa (Fig. 2A) and the NIH 3T3 (data not shown) cell lines. However, no dominant-negative effects were observed in the presence of the Egr2 SR/DY mutant, suggesting that the effect depends on a transcription factor that is expressed in Schwann cells but not in the heterologous cell lines tested.

Our previous studies indicated potential binding sites for Sox10 within the *Mpz* intron element (24). Sox10 is specifically expressed in Schwann cells and other neural crest-derived tissues (20, 33) and is required for embryonic expression of *Mpz* (37). To evaluate whether the Schwann cell-specific effects of these dominant-negative mutants were a consequence of Sox10 expression, the dominant-negative effect was tested in the B16/F10 melanocyte cell line, previously shown to express Sox10 (18). B16/F10 melanocytes were cotransfected with the *Mpz* intron element reporter or the reporter containing Egr2 binding sites. The dominant-negative mutant (SR/DY) reduced activation of the *Mpz* intron reporter by wild-type Egr2 (Fig. 2B), whereas the reporter containing four consensus Egr2 binding sites is unaffected in the presence of the Egr2 mutant (data not shown). These data suggested that Sox10 is required for the dominant-negative effect of Egr2 mutants.

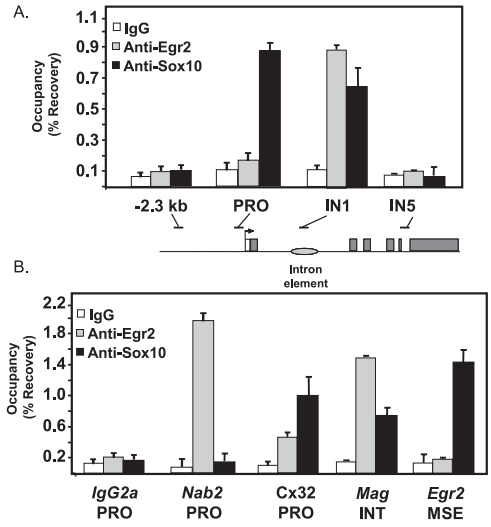

FIG. 3. Sox10 binds to the *Mpz* intron element in the S16 Schwann cell line. Formaldehyde cross-linked chromatin was prepared from S16 cells and immunoprecipitated with antibodies for Sox10, Egr2, or rabbit IgG as a negative control. Purified DNA fragments were analyzed by quantitative PCR using primers specific to the sites listed beneath each panel. Egr2 and Sox10 occupancy is expressed as the amount of DNA recovered relative to that in the input sample. (A) Amplicon positions are indicated on the diagram of the *Mpz* locus. Primer designations: −2.3 kb, 2.3 kb upstream from the transcription start site; PRO, *Mpz* promoter; IN1, *Mpz* first intron; IN5, *Mpz* intron 5. (B) Primers for the *IgG2a* promoter, *Nab2* promoter, *Cx32* promoter, *Mag* intron, and *Egr2* MSE were used to detect binding of Sox10 and Egr2 to these sites. These data are representative of the Egr2 and Sox10 binding enrichments observed in triplicate quantitative PCR assays from two independent experiments.

Sox10 binds to the *Mpz* intron element in vivo. Given the sensitivity of the *Mpz* intron reporter to dominant Egr2 mutants in Sox10-expressing cells (Schwann cells and melanocytes), regulation of this element by Sox10 was examined in greater detail. A conserved Sox10 dimeric binding site within 50 bp of the Egr2 site was identified by bioinformatics analysis (24). Interestingly, the inverted orientation of the two Sox10 sites is similar to that of previously characterized Sox10 binding sites in the *Mpz* (37, 38, 41) and *Cx32* (4) promoters. In order to determine whether Sox10 binds to the *Mpz* intron element in vivo, we carried out a ChIP assay, which employs formaldehyde to covalently cross-link DNA with associated proteins. The assay was first performed with the S16 Schwann cell line, which exhibits high levels of myelin gene expression similar to those in myelinating Schwann cells (12). After sonication, cross-linked chromatin was immunoprecipitated with anti-Sox10 or anti-Egr2 antibodies or with rabbit polyclonal immunoglobulin G (IgG) as a negative control. Following washing and reversal of cross-links, primer pairs located along the *Mpz* locus were used to detect the ChIP-enriched DNA by quantitative PCR. A primer set targeted to the *Mpz* first intron showed significant binding of both Sox10 (~6-fold) and Egr2 (~8-fold) compared with that of the control immunoprecipitate. In contrast, minimal binding was detected at the internal control sites at intron 5 of *Mpz* as well as 2.3 kb upstream of the transcription start site (Fig. 3A). An rVISTA homology search (27) indicated other potential Sox10 sites within the *Mpz* locus (e.g., IN5), but

binding of Sox10 to this site was not detected, suggesting that not all potential Sox10 sites are bound in vivo. As an additional negative control, the same samples were amplified using primers specific to the promoter of a silent Ig gene (*IgG2a*), with similar results (Fig. 3B).

Because of recent analyses demonstrating a physical interaction between Egr2 and Sox10 (50), we tested whether Egr2 and Sox10 binding are both localized at any site where either factor binds. Previous studies have indicated Egr2 sites in the *Nab2* promoter (23), and Sox10 sites have been defined in the *Mpz* promoter (37, 38, 41). Both Egr2 and Sox10 sites have been characterized in the *Connexin-32* (*Cx32*) promoter as well (4, 14, 30). As shown in Fig. 3B, substantial binding of both Sox10 and Egr2 was observed on the *Cx32* promoter. In contrast, there was preferential association of Sox10 at the *Mpz* promoter (Fig. 3A), and conversely, only Egr2 binding was detected at the *Nab2* promoter, indicating that Egr2 and Sox10 binding do not always colocalize.

Two other potential sites of Sox10 activity were analyzed by ChIP assays. Transgenic analysis identified an element (myelinating Schwann cell element [MSE]) required for expression of *Egr2* in myelinating Schwann cells (10). A recent study showed that this element contains several monomeric Sox10 binding sites, and Sox10 can cooperatively activate this element together with Oct6 in transfection assays (8). The ChIP assays in Fig. 3B revealed specific binding of Sox10, but not Egr2, to the MSE in the S16 cell line. Second, *Mag* expression is dependent on Egr2 activity (16, 32, 36, 45), and the ChIP analysis was used to examine a conserved Egr2 binding site within the *Mag* second intron (16). Interestingly, a bioinformatics analysis identified a dimeric Sox10 site approximately 50 bp upstream of the Egr2 binding site with the following sequence: AACAGGAC TCTTTTGTA. The results revealed specific binding of both Egr2 and Sox10 to the *Mag* intron site compared to that of the control immunoprecipitate (Fig. 3B), providing the first evidence for direct regulation of *Mag* expression by Sox10.

The importance of Sox10 during the embryonic specification of Schwann cells has been well established (5). Although Sox10 expression in Schwann cells is maintained through adulthood (20), its functional role during myelination in postnatal development is less clear. Sox10 and Egr2 are coexpressed in myelinating Schwann cells (1), along with high levels of *Mpz* expression (52), and *Mpz* expression in Schwann cells is dependent on both Egr2 and Sox10 (22, 24, 37, 45, 53). Therefore, ChIP assays for binding of Sox10 to *Mpz* were also performed with myelinating sciatic nerve. Freshly harvested rat sciatic nerves at postnatal day 10 were homogenized and incubated in 1% formaldehyde and then processed as described above. P10 was chosen since *Mpz* and other myelin genes are highly induced by this time point (42). Similar to the results obtained for the S16 cell line, specific binding of Sox10 was detected at the promoter and first intron element but not at other sites in the *Mpz* locus (Fig. 4A, −2.3 kb and IN5). The results indicate that Sox10 binds to the *Mpz* intron element, both in the S16 cell line and in sciatic nerve during myelination. In addition, these data provide the first direct demonstration of binding of Sox10 to the *Egr2*, *Cx32*, and *Mag* genes during myelination.

Conserved Sox10 sites are required for activation through the *Mpz* intron element. As the resolution of the ChIP assay

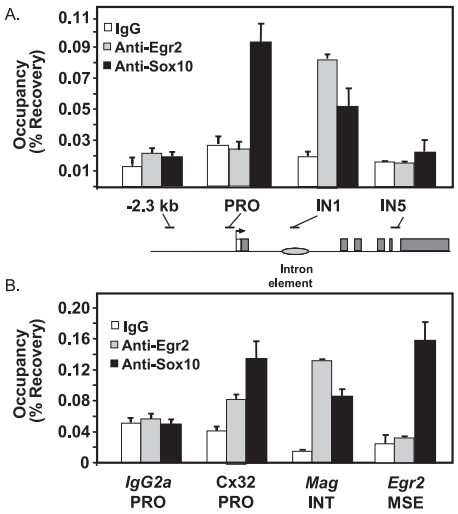

FIG. 4. Sox10 binds to the *Mpz* intron element in myelinating sciatic nerve. ChIP assays were performed by immunoprecipitating formaldehyde cross-linked chromatin prepared from sciatic nerves pooled from ~10 rat pups at postnatal day 10, using antibodies for Sox10, Egr2, or rabbit IgG as a negative control, as described for Fig. 3. (A) Amplicon positions are indicated on the diagram of the *Mpz* locus. Primer designations: −2.3 kb, 2.3 kb upstream from the transcription start site; PRO, *Mpz* promoter; IN1, *Mpz* first intron; IN5, *Mpz* intron 5. (B) Primers for the *IgG2a* promoter, *Cx32* promoter, *Mag* intron, and *Egr2* MSE were used to detect binding for Sox10, Egr2, or the IgG control antibodies. These data are representative of quantitative PCR assays performed in triplicate with two independent sets of pooled rat sciatic nerves at P10.

does not permit precise localization of Sox10 binding sites, we mutated the dimeric binding site to a nonfunctional Sox10 site as previously defined (4). The wild-type and mutant reporter constructs were transfected in HeLa cells with Sox10, Egr2, or both. As shown previously (24), Egr2 and Sox10 individually as well as synergistically activate the *Mpz* first intron element (Fig. 5A) in HeLa cells. On the reporter containing the Sox10 site mutations, however, Egr2-dependent transactivation was unaltered, but Sox10-dependent transcription—as well as synergy between Sox10 and Egr2—was abolished. Therefore, the two binding sites are essential for Sox10 activation of the *Mpz* intron element.

To test for direct binding of both Egr2 and Sox10 to these binding sites, EMSAs were performed using a fragment of the *Mpz* intron that contains the Egr2 and Sox10 binding sites (Fig. 5B). Purified Egr2 binds its conserved site in the *Mpz* intron element, and this binding is lost when the Egr2 site is mutated. In contrast, no binding of purified Sox10 protein alone was observed, even with increasing amounts (up to 8-fold) of Sox10 (Fig. 5C). It is important to note that these studies were carried out using full-length Sox10, which has been previously shown to have a lower DNA-binding affinity than truncated versions that have been used for in vitro binding studies (21, 38). However, in the presence of Egr2, Sox10 forms a slower-migrating complex that requires intact Egr2 and Sox10 binding sites. These results suggest that Egr2 facilitates binding of Sox10 to the *Mpz* intron element and are consistent with a proposed model for Sox10 synergy in which interactions between Sox factors and partner proteins are required to stabilize the bind-

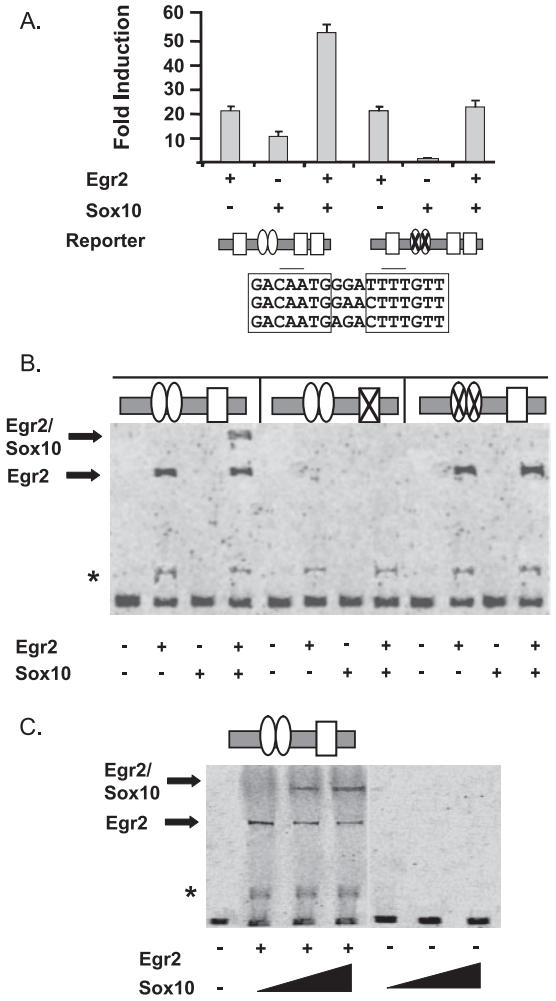

FIG. 5. Mutagenesis of Sox10 binding sites disrupts synergistic activation of the *Mpz* intron element by Egr2/Sox10. The two Sox10 sites in the *Mpz* intron element reporter were mutated by site-directed mutagenesis. The *Mpz* intron reporters (wild type and Sox10 mutant) were cotransfected in HeLa cells with expression plasmids for Egr2 (50 ng) and/or Sox10 (100 ng). The putative Sox10 and Egr2 binding sites are indicated with ovals and squares, respectively. The X in the diagram represents mutated Sox10 sites. The sequence alignment shows conservation of the Sox10 binding sites in mouse, human, and rat, and mutated bases are indicated by lines over the sequence. Induction (*n*-fold) is calculated relative to the activity of each reporter alone. (B) Mobility shift assays for Egr2 and Sox10 binding were performed using a fragment from the *Mpz* intron containing the two Sox10 sites and the second Egr2 site. These fragments either were wild type or had mutations in the Sox10 or Egr2 sites (indicated by X). Recombinant Egr2 and FLAG affinity-purified Sox10 were used. A faster-migrating, nonspecific band (asterisk) copurified with recombinant Egr2 but was unaffected by mutation of the Egr2 site. (C) Increasing amounts of purified Sox10 (8-fold range) were incubated in the presence or absence of Egr2 with the wild-type *Mpz* intron probe.

ing of the Sox proteins to their target sites (reviewed in references 17, 48, and 49).

Sox10 is required for the activity of dominant Egr2 mutants. Previous analysis has suggested that the Egr2 mutants act by interfering with a factor required for Egr2 activation of specific genes (32), and we tested whether Sox10 was the targeted factor by using the *Mpz* intron element reporter in which the

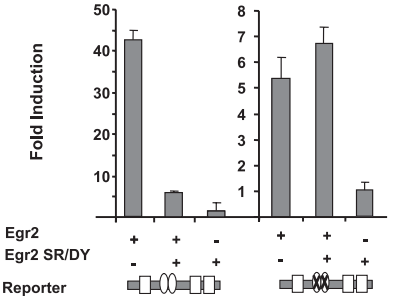

FIG. 6. Mutation of Sox10 binding sites abrogates the dominant-negative effect of a dominant Egr2 mutant. Primary rat Schwann cells were cotransfected with the *Mpz* intron reporters (wild type and Sox10 mutant) along with expression plasmids for Egr2 (50 ng), the Egr2 (SR/DY) mutant (50 ng), and Sox10 (100 ng). Induction is calculated relative to the luciferase activity of each reporter alone. Means and standard errors are representative of two independent assays performed in duplicate.

Sox10 sites are mutated. If the neuropathy-associated mutants no longer act in a dominant-negative manner on the mutant reporter, it follows that binding of Sox10 to that site is targeted by the Egr2 mutants. Primary rat Schwann cells were cotransfected with Egr2 and the wild-type or Sox10 mutant reporter, along with the dominant-negative mutant (SR/DY). Compared to that of the wild-type reporter, Egr2 activation of the reporter with mutated Sox10 sites was diminished (Fig. 6), but the dominant-negative mutants had no effect on the residual activation. Since mutating Sox10 sites reduces Egr2 activation in Schwann cells but not in HeLa cells (Fig. 5), it appears that endogenous Sox10 facilitates activation of the element by Egr2 in Schwann cells. Importantly, mutation of the Sox10 binding sites abrogates the dominant-negative effects of the neuropathy-associated Egr2 mutant.

As an independent test of whether Sox10 binding is targeted by dominant Egr2 mutants, we determined whether overexpression of Sox10 would ameliorate the dominant-negative effects of the Egr2 mutants on the *Mpz* intron element. Primary rat Schwann cells were transfected with the wild-type intron reporter along with Egr2, the dominant-negative mutant, and Sox10 (Fig. 7). While the Egr2 mutant greatly reduced the activity of the reporter in the presence of Egr2 from 43-fold to 5-fold, this was partially rescued back to 20-fold by the addition of exogenous Sox10. As a negative control, we tested whether activity would be rescued by the related SoxC protein Sox11, which is a potent transactivator (19) that nonetheless is unable to activate endogenous *Mpz* expression (37). As shown in Fig. 7, Sox11 fails to rescue the decreased promoter activity in the presence of dominant-negative Egr2. Sox10 expression had little effect in the absence of dominant-negative Egr2, indicating that endogenous Sox10 levels in primary Schwann cells were sufficient for full activation of the *Mpz* intron element.

Dominant-negative mutants cause specific loss of Sox10 binding at the *Mpz* intron element. Based on our results, we wished to test whether expression of dominant Egr2 could affect binding of Sox10 and/or Egr2 to the *Mpz* intron element. As the S16 cell line exhibits high levels of *Mpz* expression comparable to those for sciatic nerve (12), S16 cells were infected with an Ad expressing the neuropathy-associated mu-

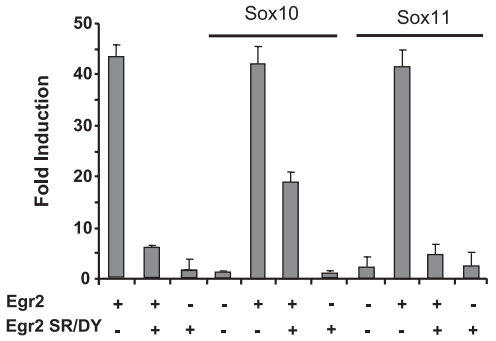

FIG. 7. Overexpression of Sox10 rescues the dominant-negative effect of a dominant Egr2 mutant. Primary rat Schwann cells were cotransfected with the wild-type *Mpz* intron reporter along with Egr2 (50 ng), Egr2 (SR/DY) mutant (50 ng), Sox10 (100 ng), and Sox11 (100 ng) expression plasmids. Induction is calculated relative to the luciferase activity of the reporter alone. Means and standard errors are representative of two independent assays performed in duplicate.

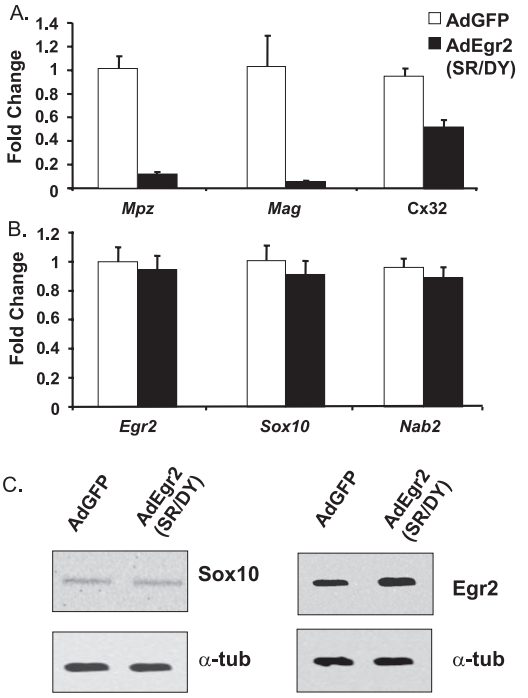

FIG. 8. A dominant-negative Egr2 mutant downregulates expression of myelin genes. S16 Schwann cells were infected with Ad expressing either GFP or the dominant Egr2 mutant (SR/DY) and harvested 48 h postinfection. Relative levels of gene expression for *Mpz*, *Mag*, and *Cx32* (A) and *Egr2*, *Sox10*, and *Nab2* (B) were determined by quantitative PCR and normalized to the level of 18S rRNA. Induction (*n*-fold) is indicated relative to the level found in the untreated sample, which was set as 1 for each gene. Quantitative PCR experiments were performed in triplicate, and the standard errors are indicated. The results are representative of two independent experiments. (C) The immunoblot shows lysates of S16 cells infected with Ads expressing the indicated proteins. Blots were probed with antibodies directed against Sox10, Egr2, and α-tubulin as a loading control.

tant (AdEgr2 SR/DY) or a control virus expressing GFP (AdGFP). The mRNA levels of *Mag* and *Mpz* were greatly reduced (23- and 8-fold, respectively), and *Cx32* levels were modestly affected (Fig. 8A). Ad-mediated expression of wild-type Egr2 did not have any negative effect on *Mpz* expression levels (data not shown). As recent reports have indicated that Sox10 regulates Egr2 expression through the *Egr2* MSE (8), we determined whether endogenous *Sox10* or *Egr2* expression was altered in the presence of the Egr2 mutant. When primers specific for endogenous *Egr2* or *Sox10* were used, no changes in expression for either gene were observed (Fig. 8B). Similarly, protein levels for Sox10 are unchanged in the presence of mutant Egr2, and the overall levels of Egr2 protein (endogenous wild type and exogenous mutant) are only modestly increased compared to those for the control infection (Fig. 8C). We further tested whether the dominant Egr2 mutant affected only genes in which Egr2 and Sox10 were bound to the same genomic regions. Consistent with the lack of Sox10 binding at the *Nab2* promoter as well as transient transfection assays (Fig. 1D and 3B), no changes in *Nab2* expression were observed (Fig. 8B).

To test the effect of a dominant Egr2 mutant on binding of endogenous Egr2 and Sox10, ChIP assays were performed with S16 samples infected with an Ad expressing Egr2 (SR/DY). The SR/DY mutant retains some affinity for Egr2 binding sites in vitro (47), and therefore, it is possible that overexpression could cause differential Egr2 binding within the intron. However, no significant change in binding of Egr2 to the endogenous *Mpz* intron element was observed compared to that for the control infection (Fig. 9A). Interestingly, binding of Sox10 to the *Mpz* intron element was significantly reduced by expression of the Egr2 SR/DY mutant (Fig. 9A). In contrast, the *Mpz* promoter and the *Egr2* MSE show no change in Sox10 binding with the Egr2 mutants, suggesting that overall Sox10 levels were unaffected. As negative controls, the *IgG2a* (Fig. 9A) and *Mpz* −2.3 kb (data not shown) regions were negative for both Egr2 and Sox10 binding in the ChIP assays.

Since Egr2 activation of *Mag* and, to a lesser extent, *Cx32* is inhibited by expression of dominant-negative Egr2 (Fig. 8A) (32), these sites were also examined in the ChIP assays. The results revealed a similarly decreased binding of Sox10 to the *Mag* intron and *Cx32* promoter compared to that for the control (Fig. 9B). The analyses of the *Mpz*, *Mag*, and *Cx32* genes demonstrate that dominant-negative Egr2 mutants selectively interfere with Sox10 binding at sites where Egr2 is colocalized.

To test the effects of the dominant Egr2 mutant on Egr2 and Sox10 binding in vitro, mobility shift assays for Egr2 and Sox10 binding were performed again with the same fragment of the *Mpz* intron element used previously (Fig. 5). As shown, binding of wild-type Egr2 and Sox10 forms a supershift complex (Fig. 9C). In the presence of Egr2 (SR/DY), Egr2 binding is unaffected, but the complex is abrogated in a concentration-dependent manner (Fig. 9C). Consistent with the ChIP results, binding by the Egr2 (SR/DY) mutant alone was not observed (data not shown). These results suggest that the Egr2 mutant dominantly inhibits Egr2-dependent binding of Sox10 to the *Mpz* intron element.

Interaction of Egr2 with Sox10 requires the N terminus of Egr2. The results for the previous experiments strongly suggest that there is a physical interaction between Egr2 and Sox10 that facilitates binding of Sox10 to the *Mpz* intron. Previous work had shown a weak physical interaction between the DNA-binding domains of Egr2 and Sox10 (50). Since these

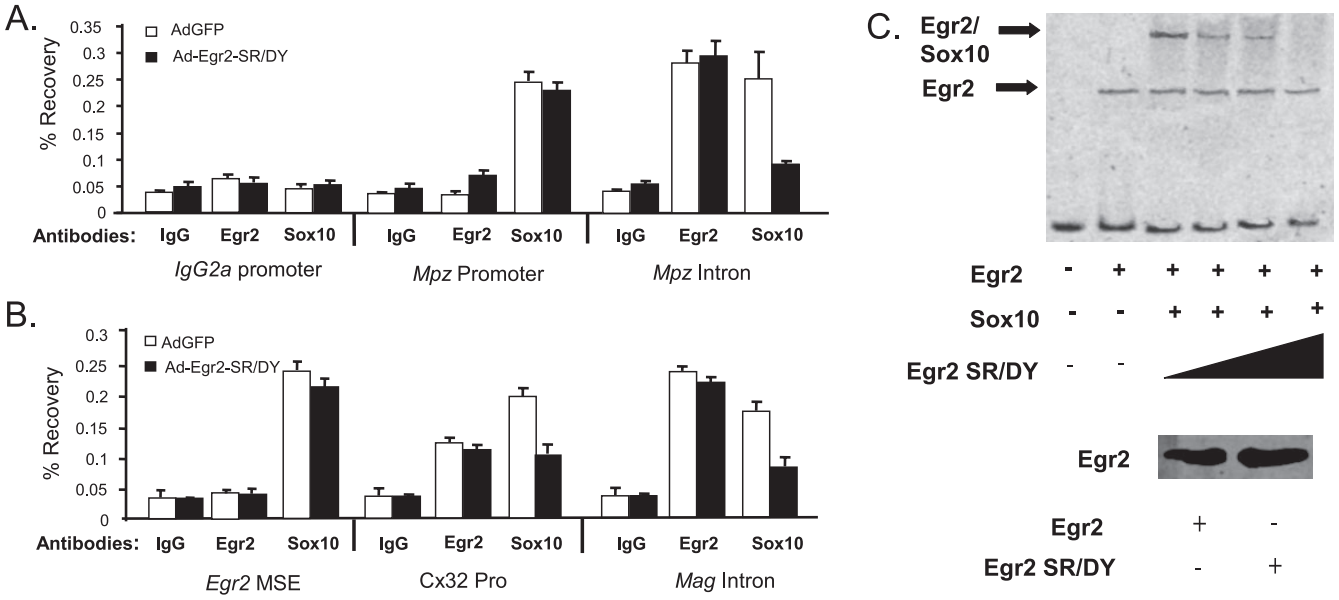

FIG. 9. A dominant-negative Egr2 mutant attenuates Sox10 recruitment to the *Mpz* intron element. S16 cells were infected with Ad expressing either the Egr2 (SR/DY) mutant or a GFP control virus as described for Fig. 8. Formaldehyde cross-linked chromatin was immunoprecipitated using antibodies for Sox10, Egr2, or rabbit IgG as a negative control. Egr2 and Sox10 occupancy is expressed as the percent recovery relative to the input sample level, which was determined by quantitative PCR using primers targeted to the *IgG2a* promoter, *Mpz* intron, and *Mpz* promoter (A) and the *Egr2* MSE, *Cx32* promoter, and *Mag* intron (B). These data are representative of two independent experiments with quantitative PCR assays performed in triplicate. (C) Mobility shift assays for Egr2 and Sox10 binding were performed using a fragment from the *Mpz* intron as described for Fig. 5, with inclusion of the dominant Egr2 (SR/DY) mutant protein. All proteins were produced by in vitro transcription/translation and purified using the six-His tag (wild type and mutant Egr2) or the Flag tag (Sox10). An immunoblot using an anti-Egr2 antibody (lower panel) shows the relative levels of wild-type Egr2 and mutant Egr2 (SR/DY) proteins (in the mobility shift assay using the lowest level of mutant protein [lane 3]).

assays had been performed with truncated proteins, we tested whether the interaction of full-length Sox10 with Egr2 would be stronger with the addition of the full-length N-terminal region of Egr2. Two recombinant proteins, full-length Egr2 and a truncated form containing the DNA-binding domain (Δ1-180), were produced and mixed at approximately equal proportions with purified Flag-tagged Sox10. As shown in Fig. 10, the immunoprecipitation assay revealed a low level of interaction between Sox10 and Egr2 Δ1-180, but the interaction with full-length Egr2 was considerably stronger, suggesting that the Egr2 N terminus is required for full interaction with Sox10. The truncated version of Egr2 was tested for its ability to facilitate Sox10 binding in the previously described EMSA system (Fig. 10). Egr2 Δ1-180 was able to bind its cognate site in the first intron of *Mpz* but was unable to stimulate Sox10 binding. Therefore, interaction of the Egr2 N terminus with Sox10 is required to stimulate binding of Sox10 to the *Mpz* intron element.

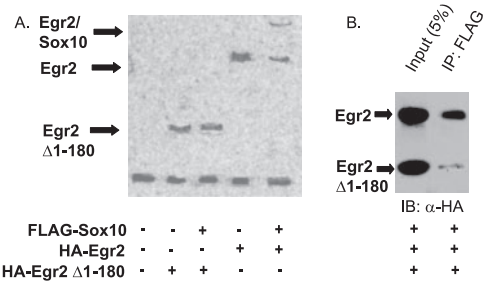

FIG. 10. The N terminus of Egr2 is required to facilitate binding of Sox10 to the *Mpz* intron element. (A) Mobility shift assays for binding of Sox10 to the *Mpz* intron fragment were performed in the presence of either wild-type Egr2 or Egr2Δ1-180 (lacking the N-terminal domain of Egr2). (B) Purified Egr2 and Egr2Δ1-180, each containing an N-terminal HA epitope, were mixed and incubated with FLAG-Sox10. Sox10 was immunoprecipitated (IP) using an anti-FLAG affinity resin, and pulldown of Egr2 was detected using an anti-HA (α-HA) antibody. The input lane represents 5% of the amount of lysate used for the binding assay. IB, immunoblot.

DISCUSSION

Sox10 is required for specification of the Schwann cell lineage (5), and mutations of Sox10 have been associated with demyelinating peripheral neuropathies (15). However, the lack of Schwann cells in the Sox10 knockout had raised the formal possibility that Sox10 might not be required for later regulation of myelin gene expression in postnatal development, even though sequence analyses and transfection assays have identified potential Sox10 binding sites in the *Mpz* and *Cx32* promoters (4, 37, 38) as well as an element controlling Egr2 expression during myelination (8). Our results have localized a novel site of Sox10 action within the conserved first intron element of the *Mpz* gene (24). Importantly, the ChIP assays performed with myelinating sciatic nerve provide the first demonstration of direct binding of Sox10 to the *Mpz* gene. Overall, our results indicate that the Sox10 protein is involved not only in specification of myelin gene expression in embryonic

Schwann cells (5) but also in maintenance of myelin gene levels in myelinating Schwann cells.

Expression of dominant, neuropathy-associated Egr2 mutants inhibits induction of myelin genes by wild-type Egr2, with the largest effect on *Mpz* expression (32). Although these observations provided the first molecular assay for the dominant nature of the Egr2 mutants, it was puzzling that regulation of the *Mpz* promoter was not similarly affected. Analysis of the *Mpz* intron element has revealed that its activation by Egr2 is uniquely sensitive to the dominant mutants. Importantly, other Egr2-dependent promoters are unaffected, indicating that mutants do not disrupt all Egr2-dependent transcription (e.g., by sequestering CBP/p300).

Several lines of evidence indicate that Sox10 activation through the *Mpz* intron element is targeted by dominant-negative Egr2 mutants. First, the dominant-negative effect was observed only in Schwann cells and a melanocyte cell line that also expresses Sox10 (18, 41). Second, mutation of Sox10 sites in the intron element leads to diminished Egr2 activation, and importantly, the residual activation is no longer sensitive to the dominant-negative effect. Third, the reduced Egr2 activation of the intron element in the presence of a dominant Egr2 mutant can be rescued by overexpression of Sox10. Finally, ChIP analysis demonstrated that expression of the dominant Egr2 SR/DY mutant causes specific loss of Sox10 binding at the *Mpz* intron element. Together, these data support the surprising finding that dominant Egr2 mutants exert a dominant-negative effect on Sox10 binding rather than Egr2 activity itself.

Several mechanistic studies have indicated that the binding/activity of Sox10 and related family members are largely dependent on physical interaction with specific partner proteins (reviewed in references 17, 48, and 49). Accordingly, mobility shift assays using full-length Egr2 and Sox10 proteins revealed that binding of Sox10 to the *Mpz* intron element is dependent on binding of Egr2 to the adjacent site (Fig. 5). Moreover, the previously described weak interaction between the DNA-binding domains of Egr2 and Sox10 (50) is further stabilized by inclusion of the Egr2 N terminus, and this domain is also required for facilitated binding of Sox10 (Fig. 10). Although the spacing between Egr2 and Sox10 sites within the *Mpz* intron (~50 bp) would seem prohibitive for Egr2/Sox10 interactions, a similar spacing has been previously reported for Sox10 and Egr2 at the *Cx32* promoter as well as the enhancer for neural crest expression of Egr2 (4, 9). Indeed, the appropriate spacing between Egr2 and Sox10 sites is likely to be critical, as previous reporter studies showed a mutually inhibitory effect when consensus Egr2 and Sox10 sites were placed in close proximity (20). Interestingly, the facilitation of Sox10 binding by Egr2 in vitro was also disrupted by inclusion of a dominant Egr2 mutant (Fig. 10), indicating that these mutants disrupt Egr2-dependent binding of Sox10 to the *Mpz* intron element.

Interestingly, ChIP assays indicated that Sox10 binding sites in the *Egr2* MSE or the *Mpz* promoter were unaffected in the presence of the Egr2 mutant. These data were consistent with the observations that activation of the *Mpz* promoter was not affected by dominant Egr2 mutants and that only a subset of Egr2 target genes were affected by expression of a dominant Egr2 mutant (32). This could reflect differential affinities of these sites for Sox10. Alternatively, since Sox10 interacts promiscuously with a variety of DNA-binding proteins (50), other factors, such as Sp1, ZBP-99, and POU domain proteins (6, 8, 41), may be involved in stabilizing Sox10 at these other sites so that they are unaffected by the dominant Egr2 mutants. These results also provide a rationale for the lack of hindbrain or bone phenotypes associated with the Egr2 mutants (46, 47), as the dominant mutants may affect only genes that are coregulated by Egr2 and Sox10 in myelinating Schwann cells.

Since even haploinsufficiency of *Mpz* expression causes a peripheral neuropathy in mice (28), the proposed mechanism is expected to play a major role in the mechanism by which dominant Egr2 mutants disrupt peripheral myelination. However, other mechanisms may also play a role. While expression of wild-type Egr2 normally causes cell cycle arrest (35), recent studies have shown that the (SR/DY) mutant induces Schwann cell proliferation through upregulation of cyclin D1 and reduction of the cell cycle inhibitor p27 (2). Nonetheless, the finding that three neuropathy-associated Egr2 mutants reduced *Mpz* expression suggests that downregulation of myelin gene expression is a common mechanism resulting in neuropathies.

Given the binding of Egr2 and Sox10 to the regulatory elements of *Cx32* and *Mpz*, it is tempting to consider that other members of the myelination program are jointly regulated by Sox10 and Egr2. Indeed, our ChIP analysis identified *Mag* as a potential target of coregulation by the two transcription factors. Previous studies had also shown that myelin genes such as *Mag*, *Pmp22*, and *Periaxin* were downregulated in the presence of the neuropathy-associated Egr2 mutants (32), and Sox10 binding at the *Mag* intron is also reduced with expression of the Egr2 mutant. Further identification of Sox10/Egr2 responsive regions in other myelin genes may reveal additional targets for dominant Egr2 mutants.

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