Induction of myelin genes occurs around birth in the last stage of Schwann cells differentiation and is reactivated in case of nerve injury. Previous studies showed that activation of the gp130 receptor system, using as ligand interleukin-6 fused to its soluble receptor (IL6RIL6), causes induction of myelin genes such as myelin basic protein (MBP) and myelin protein zero (Po) in embryonic dorsal root ganglia Schwann cells. We also reported that in murine melanoma B16/F10.9 cells, IL6RIL6 causes a shut-off of melanogenesis mediated by a down-regulation of the paired-homeodomain factor Pax3. The present work demonstrates that these IL6RIL6-treated F10.9 cells undergo transdifferentiation to a myelinating glial phenotype characterized by induction of the transcriptional activities of both Po and MBP promoters and accumulation of myelin gene products. For both Po and MBP promoters, a repression by Pax3 and stimulation by Sox10 can be demonstrated. Because after IL6RIL6-treatment, Pax3 disappears from the F10.9 cells (as it does in mature myelinating Schwann cells) whereas the level of Sox10 rather increases, we modulated the relative level of these factors and show their involvement in the induction of myelin gene expression by IL6RIL6. In addition, however, we show that a C/G-rich CACC box in the Po promoter is required for activation by IL6RIL6, as well as by ectopic Sox10, and identify a Kruppel-type zinc finger factor acting through this CACC box, which stimulates Po promoter activity.

Axonal myelination is a function of specialized glial cells, oligodendrocytes in the brain and myelinating Schwann cells (SC) in peripheral nerves, whose impairment in demyelinating diseases affects nerve function and integrity. Maturation of cells producing myelin occurs around birth and needs to be reactivated for repair of nerve injury (1). The developmental stages from neural crest-derived precursors to mature myelinating SC were defined by specific gene markers (see Refs. 2–5 for reviews). At early stages, embryonic SC express low affinity nerve growth factor receptor, neural cell adhesion molecule (N-CAM), glial fibrillary acidic protein (GFAP), the transcription factors paired homeodomain Pax3, and later POU domain Octamer-6 (Oct-6)/suppressed cAMP-inducible POU (SCIP). This phenotype also characterizes the adult non-myelinating SC and reappears after nerve injury. Maturation of myelinating SC is marked by disappearance of these early markers, including Pax3, and the induction of myelin genes such as myelin basic protein (MBP) and protein zero (Po or myelin protein zero). Promoters driving glial-specific expression of Po (6–8) and of MBP (9–14) were identified along with regulatory transcription factors (see Ref. 15 for review). Thus, the high mobility group (HMG) domain Sox10, already present in early neural crest cells, activates the Po promoter (16). Krox20/Egr2, a late marker of myelinating SC differentiation required for myelination (17), stimulates expression of several myelin genes (18, 19). Conversely, Pax3 represses expression of MBP (20), and so does SCIP (21), in line with the need for of Pax3 and SCIP to decrease during the terminal differentiation of myelinating SC.

Among extracellular factors acting on SC, only a few could be shown to activate myelin gene expression. The main SC growth factors, neuregulins nerve-derived factor (NDF)/glial growth factor (GGF) (22), stimulate proliferation at early SC differentiation stages but have negative effects on the induction of MBP and Po genes (23). Similarly, fibroblast growth factor or transforming growth factor-β inhibit Po gene expression (24). Axonal contacts are thought to provide positive stimuli for myelin gene expression in SC cultures (25) and in transected nerves (26). The molecules mediating these axonal cues are not well known. Intraaxonal cyclic AMP elevation (e.g. by forskolin) induces Po and MBP (25, 27) whereas it represses Pax3 (20). Activating effects of forskolin were seen on the promoters of the genes encoding myelin proteins Po (6), MBP (28, 29), and peripheral myelin protein-22 (PMP-22) (30). Hormones, such as progesterone and glucocorticosteroids, also stimulate Po and PMP-22 promoter activities (31, 32). A third group of factors are IL-6 family cytokines that activate the gp130 receptor system. As a prototype of this large family (see Ref. 33 for review), we have used a recombinant protein IL6RIL6 resulting from the fusion of IL-6 to its soluble IL-6 receptor (sIL-6R) that activates the gp130 signaling pathway in many cell types and has a high affinity for gp130 (34, 35). We showed that this gp130 activator potently stimulates the expression of MBP and Po mRNAs and proteins in cultures of mouse E14 embryonic dorsal root ganglia (DRG), as well as in derived SC (36, 37). IL6RIL6 strongly down-regulated the expression of Pax3 in these embryonic cells. Moreover, IL6RIL6 enhanced in vivo the number of myelinated fibers in regenerating sciatic nerve (37). Others observed that IL6RIL6 induces MBP RNA in brain.
cells, as well.2 The physiological relevance of these data is supported by the fact that in mice the conditional inactivation of gp130 after birth causes loss of myelin sheaths and SC defects in peripheral nerves (38).

We show here another system in which the gp130 activator IL6RIL6 switches on the expression of myelin genes and causes transdifferentiation of melanoma cells toward a glial phenotype. The murine melanoma B16/F10.9 cells undergo terminal growth arrest when exposed to the combination of IL6 with its agonistic soluble IL-6 receptor (39) or to the IL6RIL6 chimera (34). There is a silencing of the melanogenic pathway because of a profound reduction in Pax3, which then down-regulates the microphthalmia-associated transcription factor (MITF) gene and, in turn, the tyrosinase activity (40). The present work demonstrates that IL6RIL6 subsequently induces expression of the myelin Po and MBP mRNAs through promoter-mediated transcriptional activation. This melanoma provides a new model to study the transcription factors involved in the regulation of myelin gene expression and their induction by IL6-family cytokines.

MATERIALS AND METHODS

Cell Cultures and Cytokines—Murine B16 melanoma metastatic clone cells (41, 42) were cultured as a monolayer at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium with 8% fetal calf serum (Biolas, Bet Ha-Emek, Ness Ziona, Israel), supplemented with glucose, penicillin, and streptomycin. Cells were subcultured every 3 days at 10–30% confluency. Fused IL6RIL6 chimera was produced and used as described (34, 35) using mammalian Chinese hamster ovary cells and immunoaffinity purification of the secreted 85-kDa protein (Inter-at 10

Activation of Myelin Genes Po and MBP by IL6RIL6

mid (Clontech) cloned in pEFIRE-puro, using primers of before

The clones were screened for rtTA activity, with the vector pSV2Hygro, together with pBI vectors containing a bidirectional Tet-regulated element (Clontech). In the selected clone cells, we introduced the vector pSV2Hygro, together with pBI vectors downstream to the Tet-regulated element, we cloned the cDNA for the green fluorescent protein, enhanced green fluorescent protein (Clontech), either alone (control) or with Sox10 or Pax3 cDNA (in the opposite direction). F10.9-rtTA cell clones growing in the presence of hygromycin and becoming fluorescent after treatment with doxycycline (200 ng/ml) were selected. Expression of the ectopic mRNAs was measured by RT-PCR, using the vector reverse primer 5’ ACTCACCTT-GAAGTCTCG and forward primers Sox10 (AF074043; nt 924–948) or Sox11 (NM_008781; nt 880–898).

Promoter Reporter Gene Constructs—The MPB reporter plasmid pBGl1 (9) (a gift of Dr. C. Kioussi, San Diego, CA) was excised with BglII and BspEI and blunt-ended to obtain the 5′ flanking MBP gene sequence to 1320+33. The fragment was cloned in front of the luciferase coding sequence in the pGL3 basic plasmid (Promega) cut by XhoI and HindIII, blunt-ended, to create the plasmid pGL3MBP–1.3. The latter was cut with BglII (in 5′) and PstI (in the MBP sequence) and closed by ligation to create pGL3MBP–0.65 or was cut with EcoRl–1 and PooII to create pGL3MBP–105. The rat Po promoter (6) was generated by genomic PCR, using the following primers: F, 5′-GACATTATCCCCTCCATTCCCTATATTCCCG-3′; and R, 5′-GCCGACGACCGCTGCTGGGAGAAGCGG-3′. After elimination of the amplification fragment (sequence –912 + 45 relative to the start site) was cloned upstream of luciferase in pGL3 basic cut with SauI to create pGL3-Po.192. For pGL3-Po.500, Po gene sequence 500+45 was made by PCR with pGL3-Po.192 as template and primers F, 5′-GGGAGCCTAGCCAGGATCGCAGGATTCCGAGAAGCGG-3′ and R, 5′-GGGAGGATCCTTGCAGGAGGATListen to the audio file for a detailed explanation.
pared from F10.9 cells grown in 9-cm dishes for different times in the presence or absence of IL6RIL6 (500 ng/ml). The cell monolayer, washed with PBS (minus calcium, magnesium), was scraped with a rubber policeman in 2 ml of phosphate-buffered saline into Eppendorf tubes. Pellets recovered by centrifugation at 3000 rpm were frozen and stored in liquid nitrogen until use. Pellets were thawed on ice and homogenized in hypotonic Buffer A (4 volumes per volume pellet) by mixing five times with pipette, left 10 min on ice, and centrifuged for 10 min at 4 °C at 5000 rpm. The pellet compacted by a 10-g spin at 14000 rpm was resuspended in 2.5 volumes (relative to original cell pellet) of Buffer B by mixing five times with pipette, left on ice for 10 min, and centrifuged 14000 rpm for 10 min at 4 °C. The supernatants (nuclear extracts) were kept at −70 °C. Buffer A contained 10 mM Hepes, pH 7.9, 100 mM NaCl, 5% glycerol, 2 mM EDTA, 2 mM EGTA, 50 mM NaF and was completed at the last moment with 1 mM dithiothreitol, 10 mM sodium molybdate, 0.1 mM sodium orthovanadate, and a mix of protease inhibitors (Calbiochem) diluted 1/50 in Buffer A. Buffer B is the same as Buffer A with 400 mM NaCl. Complementary oligonucleotides of Po promoter 176/151 segment (see Fig. 6D) were 5′-labeled with γ³²P[ATP (10³ cpm/nmol) and poly nucleotide kinase. After annealing, the double-stranded oligonucleotide was purified on a non-denaturing 8% polyacrylamide gel. About 20,000 cpm of the oligonucleotide probe (20 fmol) was incubated with 2 μl of nuclear extracts for 20 min on ice in a final volume of 20 μl. The incubation buffer final composition was 20 mM Hepes, pH 7.9, 60 mM NaCl, 1 mM dithiothreitol, 5% glycerol, 5 mM MgCl₂, 0.1 mM ZnCl₂, and 100 μg/ml bovine serum albumin, with, respectively, 2 and 0.1 μg of poly(dG)-poly(dC) and poly(dI)-poly(dC) alternate copolymers per assay (Roche Molecular Biochemicals). For competition, 2 μM of cold wild-type 176/151 probe or oligonucleotides with mutated CACC site were used as follows: 5′-TGGTTGCTCCT-GAAGCTCTACATCCAGA-3′ (−169/−161 mutant) or 5′-TGGTTGCCCT-CCGGCCCTACCCAGA-3′ (−165/−163 mutant).

One-hybrid System—The MATCHMAKER one-hybrid system from Clontech was used according to the manufacturer’s protocols. Complementary synthetic oligonucleotides containing the CACC box sequence −174/−156 of the myelin Po promoter CTGTTCCCCACCCATCA were placed in four tandem repeats upstream of the pHis-1 and pLaZ1 plasmids. Target reporter strains of Saccharomyces cerevisiae TM4/C71 were obtained after transformation with these plasmids and tested for minimal background growth in minimal medium lacking histidine and containing calibrated concentrations of 3-amino-1,2,4-triazole (3-AT). A cDNA library (minimal length 400 bp) was prepared from RNA extracted from F10.9 melanoma cells that had been treated for 48 h with IL6RIL6. The amplified and tailed cDNAs were used for recombination-mediated cloning in yeast with the Smal-linearized pGADT7-Rec plasmid to form fusion products with the Gal4 activation domain upon transformation into the target reporter yeast strain. Large positive yeast colonies growing in the minimal synthetic dropout (SD) medium lacking histidine and leucine and supplemented with 30 mM 3-AT were selected. Plasmids were isolated from the vegetative colonies by cloning into E. coli DH5α and were tested individually in the yeast reporter strain. Screening was done in the histidine- and leucine-selective medium to form cell tracks. Concomitantly to the cell shape changes, the CNPase protein, another constant component of myelin Po, 3′′′′151 probe or oligonucleotides with mutated CACC site were used as follows: 5′-TGGTTGCTCCT-GAAGCTCTACATCCAGA-3′ (−169/−161 mutant) or 5′-TGGTTGCCCT-CCGGCCCTACCCAGA-3′ (−165/−163 mutant).

RESULTS

Induction of Myelin Gene Products following Loss of Melano- genesis in Melanoma Cells—As previously reported (40), treatment of the melanoma F10.9 cells by the gp130 activator IL6RIL6 induces a transition in morphology from epithelial-like cells to elongated cells with extended processes that align to form cell tracks. Concomitantly to the cell shape changes, the IL6RIL6-treated cells stopped releasing melanin pigment in the medium, and the rate-limiting melanogenic enzyme tyrosinase decreased. We have demonstrated (40) that the loss of tyrosinase upon IL6RIL6 treatment results from the decrease in the transcription factor MITF, decrease that is itself mainly because of the loss of Pax3 from the cells. Fig. 1A shows that following the Pax3 RNA down-regulation, there was a strong induction of MBP transcripts in the IL6RIL6-treated melanoma cells. A progressive accumulation of MBP mRNA was observed from 12 to 48 h (Fig. 1B). Transcripts for myelin Po, which is the most abundant component specifically found in the peripheral nerve myelin made by Schwann cells, were strongly induced by IL6RIL6 in the F10.9 cells starting from 24 h (Fig. 2C). The CNPase protein, another constant component of myelin (43), was similarly induced by IL6RIL6 in the F10.9 melanoma cells (Fig. 2D). There was also an induction of FMP-22 and of galactocerebroside (GalC; not shown). The F10.9 melanoma cell response to IL6RIL6 can be defined as a transdifferentiation, because the cells transit from a melanocytic phenotype, which is enhanced if the cells are treated for forskolin (40), to a phenotype characteristic of myelinating Schwann cells.

Transcriptional Activation of Myelin Po and MBP Gene Pro- moters in IL6RIL6-treated F10.9 Melanoma Cells: Roles of Pax3 and Sox10—To determine whether the induction of the MBP and Po mRNAs following IL6RIL6 treatment results from transcriptional gene activation, we cloned the 5′ flanking −1320/+33 region of the murine MBP proximal promoter (9) in front of the luciferase reporter gene. This region of the promoter confers tissue-specific expression in cell lines and in myelinating oligodendrocytes in vivo (44). Transfection of the F10.9 cells with this reporter gene demonstrated that the MBP gene undergoes a transcriptional activation of about 5-fold in response to the IL6RIL6 stimulus (Fig. 2A). For the myelin Po gene, the 5′ flanking sequences −912 to +45 of the rat gene, which confer specific expression in Schwann cells (6, 7), were cloned in the luciferase reporter gene and transfected into F10.9 cells. IL6RIL6 induced 10−15-fold the activity of this myelin Po promoter construct (Fig. 2B). Because one of the major effects of IL6RIL6 in the transdifferentiating F10.9 cells is the decrease of Pax3 protein (Fig. 2D) (40), we investigated if Pax3 may be involved in the regulation of the myelin Po gene promoter activity, as reported for the MBP gene in primary Schwann cells (20). The F10.9 cells were transfected with a Pax3 expression vector in addition to the MBP and Po promoter reporter genes. The IL6RIL6-dependent induction of the promoter activities for both MBP (Fig. 2A) and Po (Fig. 2B) was
Fig. 2. Ectopic expression of Pax3 inhibits Po and MBP promoter activities; down-regulation of Pax3 with IL6RIL6 treatment may account for promoter induction. A, F10.9 cells co-transfected with MBP promoter (–1.3kb/+33), luciferase reporter (0.5 μg), and different amounts of pcDNA3-Pax3 plasmid (with empty pcDNA3 to keep DNA constant at 3.3 μg). Promoter activity, expressed as firefly luciferase values, normalized on Renilla luciferase values, is shown at 48 h with 350 ng/ml IL6RIL6 (black bars) or untreated (open bars). B, same with Po promoter −912/+45, in the same transfection experiment. C, plot of the -fold increase by IL6RIL6 and its repression by different ratios of pcDNA3-Pax3 over the reporter plasmid in three experiments of transfection with Po −912/+45 or MBP −1.3 kb/+33 promoters. D, Western blots of nuclear extracts from F10.9 (72 h without or with IL6RIL6 treatment, 140 ng/ml) reacted successively with Sox10 antibodies and after stripping, with anti-Pax3 antibodies. E, RT-PCR for Sox10 RNA in F10.9 cells without and with IL6RIL6 for 72 h.

Inhibited upon expression of Pax3. Increasing doses of Pax3 cDNA produced parallel reductions in the effect of IL6RIL6 on the myelin Po and MBP gene promoters (Fig. 2C). Thus the repressor effect of Pax3 on the MBP gene is operating in the melanoma F10.9 cells and can now be extended to the peripheral myelin Po gene, as well. Up to 80% inhibition by Pax3 was seen with the −912/+45 Po promoter construct (Fig. 2B), and a dose-dependent decrease could also be observed with a −300/+45 Po promoter construct (data not shown). Inspection of the rat Po promoter sequence does not indicate the presence of Pax3 binding sites, leaving the possibility that Pax3 acts indirectly. In any event, this repression effect on the myelin Po and the MBP promoters strongly suggests that the down-regulation of Pax3 by IL6RIL6 contributes significantly to the induction of the myelin gene expression.

The B16/F10.9 cells express endogenous Sox10, an HMG factor found to be important in both melanocytic (45–48) and SC differentiation (15, 16, 49). An increase in Sox10 RNA was observed by RT-PCR with RNA extracted 72 h after IL6RIL6 addition to the cells (Fig. 2E). This induction was ascertained by real-time PCR (Light Cycler), which indicated an increase in Sox10 transcripts of 2- and 5.5-fold, respectively, at 48 and 72 h post-IL6RIL6 addition. The amount of Sox10 protein measured by immunoblots in nuclear extracts of F10.9 cells showed also an increase, which contrasts with the decrease in Pax3 protein (Fig. 2D). We reported before (40) that this increase in Sox10 is not seen during the first day after IL6RIL6 treatment, where Sox10 was even lower than in untreated cultures, but becomes apparent at days 2 and 3 amounting to a 50% increase in the level of Sox10 protein in the treated cell nuclei.

Co-transfections with Sox10 expression vectors showed that Sox10 is positively involved in the activation of the MBP promoter in the F10.9 melanoma cells non-treated with IL6RIL6 (Fig. 3A). In the same experiment, the Pax3 vector again strongly repressed the MBP promoter. Induction by Sox10 was observed with the three MBP constructs tested, including the shortest construct containing only the proximal −105/+33 fragment of the MBP promoter (Fig. 3A). A further shortened construct, −70/+33, failed, however, to respond to either Sox10 or IL6RIL6 (not shown). These experiments show that MBP transcription in F10.9 cells is dependent on Sox10, as found very recently (50) in oligodendrocytes by in vivo and in vitro experiments. Likewise, transfection of Sox10 into the F10.9 melanoma stimulated the Po promoter transcriptional activation in a dose-dependent manner in the absence of IL6RIL6 (Fig. 3B). At high ratios of Sox10 over the Po reporter the activation was close to that produced by IL6RIL6. A truncated form of Sox10 (E188X) was not comparably active (not shown). In the cells treated by IL6RIL6, Sox10 did not increase the Po promoter activity (Fig. 3B) and did not increase the MBP promoter activity (not shown). Hence, in these cells, Sox10 appears to be a limiting factor in the activity of the MBP and Po gene, unless the cells have been treated by the gp130 activator.

Modulation of the Concentrations of Pax3 and Sox10 in Stably Transfected F10.9 Cells Regulates the Level of Cellular Myelin Po mRNA—To further evaluate the contributions of Pax3 and Sox10 on the induction of Po mRNA, the F10.9 cells were permanently transformed with vectors containing Pax3 and Sox10 cDNAs placed under the control of a tetracycline-regulated element. The levels of Pax3 and Sox10 transcripts were measured by semiquantitative RT-PCR in the cells treated by doxycycline or IL6RIL6 or both (Fig. 4A), and in parallel we measured the amount of myelin Po mRNA. In cells that overexpressed Pax3 when treated with doxycycline, the level of Po RNA induced by IL6RIL6 was decreased by about 50% as compared with the level induced in the absence of doxycycline (Fig. 4B). The basal level of Po RNA was also very
Activation of myelin genes Po and MBP by IL6RIL6.

A

IL6RIL6

Dox.

Pax3

Sox10

mRNAs

Total

Ectopic

B

Ctrl

Sox10

Pax3

Pax3+IL6RIL6

Doxy.

Fig. 4. Modulation of Pax3 and Sox10 concentrations in F10.9 melanoma cells regulates the level of cellular myelin Po mRNA. A, clones of F10.9 cells that were stably transfected with Pax3 (left panel) or Sox10 (right panel) cDNAs placed under the control of the Tet-cycline-regulated element were treated as indicated (+) with doxycycline (1 µg/ml) or with IL6RIL6 (350 ng/ml) or with both. After 48 h, RNA was extracted from the cultures, and total cellular Pax3 or Sox10 RNA was measured by RT-PCR, as well as Pax3 or Sox10 RNAs specifically originating from the Tet-regulated ectopic genes. B, the level of cellular Po mRNA was determined by scanning RT-PCR bands intensity using Tet-regulated clones containing cDNAs for enhanced green fluorescent protein (control, open diamonds), Sox10 (open squares), or Pax3 (open triangles), cultured 48 h without and with indicated amounts of doxycycline and for Pax3 also with IL6RIL6 and doxycycline (closed triangles). The Po mRNA level in control cells treated with IL6RIL6 was taken as 100%.

Conversely, with a clone that overexpressed ectopic Sox10 in response to doxycycline (Fig. 4A), we observed an increase of Po mRNA (Fig. 4B). However, under conditions where the increase in total Sox10 RNA caused by doxycycline was comparable with that in IL6RIL6-treated cells (Fig. 4A), the increase in Po was less than the one induced by IL6RIL6 treatment (Fig. 4B). Thus, the increase in Sox10 caused by IL6RIL6 could account for part, but probably not all, of the induction of the Po mRNA by IL6RIL6 treatment. This conclusion was confirmed when we next examined the Po promoter sequences required for the transcriptional activation by IL6RIL6.

Sequences Required for the Activation of the Po Promoter by IL6RIL6: Involvement of Sox10 Sites and of a CACC Box—The rat Po promoter sequence mediating tissue-specific expression of Po contains a number of binding sites for known transcription factors (8, 16), some of which are indicated in Fig. 5A. Po promoter constructs with sequences from −912, −500 or −300 bp to +45 all gave 10–15-fold activation by IL6RIL6 (Fig. 5B). Further 5′ deletions from −300 to −136 resulted in a progressive loss of induction by IL6RIL6, most of the decrease occurring between −222 and −137 (Fig. 5C). By further deletion from −136 to −63, the basal activity of the promoter decreased steeply probably because of the loss of the two CAAT boxes (Fig. 5, A and C). Several 5′ deletions were made and inserted upstream of a minimal TK promoter. As shown in Table I, a TK construct containing the Po −300 to −137 sequence was highly inducible by IL6RIL6, although its overall activity was lower than the −300 to −30 construct. The −222/−137 segment of the Po promoter (Fig. 5A, dotted lines) contains the Sox10 sites C (−204/−198), C′ (−193/−187), and B (−147/−140). These sites, as characterized by Peirano et al. (16), bind bacterially produced Sox10 and mediate the rat Po promoter activation in cells stimulated to express Sox10 by doxycycline. We therefore examined the effect of mutating these sites as part of a series of linker scanning mutations in the −300 to −137 region (relevant mutants indicated in Fig. 5D). Mutation of the Sox10 site C′ by itself caused a 77% reduction in the IL6RIL6-induced activity of the promoter and markedly reduced the -fold induction (Table I). Mutation of the Sox10 C and B sites also reduced the induction by IL6RIL6, the reduction being greater when both sites were mutated (Table I). However, we found that additional mutations located between the Sox10 C′ and B sites also affected the activity of the Po promoter and its inducibility by IL6RIL6. In particular, mutating the C/G-rich Sp1-like site or CACC box at −168/−161 (mutation of CCCACCCC; see Fig. 5D) consistently reduced the activity and response to IL6RIL6 in the Po −300/+45, Po −222/+45, and Po TK −300/+137 constructs (Table I). The sequences at and around −168/−161 do not resemble a Sox10 site, indicating that the induction by IL6RIL6 is not only because of an effect on Sox10.

The induction of Po promoter activity by ectopic Sox10 was, as expected, abrogated by mutations of the Sox10 sites B and C.
or mutation of site C (Fig. 6A). Interestingly, the \(-168/-161\) CACC box mutation decreased the induction by ectopic Sox10 as it did for the IL6RIL6 inducer (Fig. 6A, right panel). Changing just the central CAC (\(-165/-163\)) of this element into GGG also reduced the activity of the Po promoter in response to IL6RIL6 and Sox10 (Table I), as did a mutation of \(-176/-169\), which shortens the C/G-rich arm (Fig. 6A). These results strongly suggest a model in which Sox10 acts with one or more cooperative factor binding to the CACC box and/or its C/G-rich arms.

**A Kruppel-type Factor Is Involved in the Function of the \(-168/-161\) CACC Box in the Po Promoter—Electrophoretic mobility shift assays with a \(-175/-151\) probe demonstrated binding of two slow migrating complexes (A and B) and one fast migrating complex (C), which were specifically competed by the unlabeled probe but not when that probe had the \(-168/-161\) mutation destroying the CACC box (Fig. 6B, lanes 3 versus 4). Using probes with the \(-165/-163\) CAC mutated to GGG or TTT also affected the formation of these complexes (not shown).

Although the complexes were seen with nuclear extracts of untreated cells, IL6RIL6 produced a significant change in the pattern of complex formation, with a consistent increase in complex C but a decrease in complex A (Fig. 6B, lanes 1 versus 2).

In search of a factor that could act through this element of the Po promoter, we used a one-hybrid selection approach in yeast. A cDNA library prepared from IL6RIL6-treated F10.9 cells was screened for clones encoding a protein that binds a target reporter construct containing the \(-174/-156\) sequence of the Po promoter. Screening of about \(0.5 \times 10^6\) clones, followed by re-screening under stringent conditions of yeast growth (see "Materials and Methods"), identified a clone with a 3.6-kb insert. This clone was found by sequencing to contain the entire open reading frame of a cDNA designated ZBP-99 (accession number XM_129403), and RT-PCR indicated that its corresponding mRNA was significantly increased following treatment of the F10.9 cells by IL6RIL6 (Fig. 7A).

ZBP-99 (797 amino acids) has been described (51) as a member of a class of transcription factors that contain a characteristic array of four Kruppel-type zinc fingers and bind to CACC boxes, such as that in the gastrin gene promoter whose sequence and length (CCCCCCACCCCCGCC) resembles that of the \(-171/-161\) element in the Po promoter (see Fig. 5D).

Although ZBP-99 was described as an inhibitor of transcription (51), we found that it stimulates the myelin Po promoter activity (Fig. 7B). When expressed in the F10.9 melanoma cells, the full-length ZBP-99 stimulated by itself the transcriptional activity of the Po-300 promoter, whereas a truncated form of ZBP-99 had no effect (Fig. 7B). The combination of Sox10 with ZBP-99 produced a 10.2-fold stimulation of the Po promoter, above the additional effect of each factor alone, and similar to that seen following IL6RIL6 treatment of the cells (Fig. 7B). The truncated ZBP-99, lacking its C-terminal domain, was inactive or slightly inhibitory even with Sox10 or IL6RIL6.

### Table I

| Plasmid | Luciferase, percent* | -Fold induction* |
|---------|---------------------|-----------------|
| Not treated | With IL6RIL6 | |
| Expt. 1 | | |
| TK Po-300-30 | 7.6 ± 0.5 | 100.0 ± 4 | 14.1 |
| TK Po-300-137 | 3.3 ± 0.2 | 38.4 ± 1 | 13.7 |
| TK Po-300-137 with -168/-161 mut. | 3.1 ± 0.2 | 7.5 ± 1 | 2.4 |
| TK min | 0.6 ± 0.3 | 1.4 ± 0.2 | |
| Expt. 2 | | |
| Po-300 | 9.4 ± 1.2 | 100.0 ± 4 | 10.7 |
| Po-300 Sox10 B mut. | 5.4 ± 0.3 | 44.7 ± 2 | 8.4 |
| Po-300 Sox10 C mut. | 5.9 ± 0.5 | 33.2 ± 1 | 5.7 |
| Po-300 Sox10 C’ mut. | 4.8 ± 0.2 | 23.0 ± 0.8 | 4.8 |
| Po-300 Sox10 B + C mut. | 3.0 ± 0.1 | 13.1 ± 0.4 | 4.4 |
| Po-300 with -168/-161 mut. | 4.4 ± 0.5 | 29.2 ± 1.7 | 6.7 |
| Po-300 with -165/-163 mut. | 11.7 ± 1.0 | 32.6 ± 1.8 | 2.8 |
| Po-222 | 5.6 ± 0.2 | 53.3 ± 2.2 | 9.6 |
| Po-222 with -168/-161 mut. | 1.7 ± 0.1 | 10.8 ± 0.3 | 6.6 |
| Basic pGL3 | 0 ± 0 | 0.2 ± 0.01 | |

* Normalized luciferase values expressed as percent of reference construct with IL6RIL6. The 100 percent values for Expt. 1 and 2 are 1110 and 1050, respectively (mean of triplicates).
* Fold induction calculated after subtracting the TK min and basic pGL3 values.
* Mutations (mut.) in the rat Po promoter sequence indicated in Fig. 5D.
synergism, although the effect of Sox10 was reduced (Table II). Thus, the site of Krox20 action must be elsewhere. Indeed, mutation of the upstream CCC/CACCC element (−247/−240; see Fig. 5D) reduced the effect of Krox20 more than those of Sox10 or IL6RIL6 (Table III). Another C/G-rich element, GGAGGAGG (−220/−213), also affected the response to Krox20 with less effects on the other inducers (Table III). Krox20 may, therefore, work through several distinct upstream sites. The mutation analysis further showed that the effect of IL6RIL6 on Po promoter activity correlates less with the effect of Krox20 than with the response to Sox10. We also did not find that IL6RIL6 treatment significantly changed the level of Krox20 RNA already present in these F10.9 cells (not shown). Instead, the −168/−161 CACC box appears to be the main element required, in addition to its surrounding Sox10 binding sites, for the activation of the Po promoter by IL6RIL6, as well as by Sox10 in the F10.9 melanoma cells.

**Discussion**

The gp130 activator IL6RIL6 activates myelin gene expression in embryonic Schwann cells (36, 37). We show here that IL6RIL6 induces expression of the myelin genes in the murine B16/F10.9 melanoma cells. These cells undergo a transdifferentiation, with loss of their melanocytic phenotype characterized by MITF and tyrosinase expression (40) and acquisition of a myelinating Schwann cell phenotype. Melanoma and nevus cells can exhibit such phenotypic changes in vivo (53, 54). The inducible transdifferentiation of the F10.9 cells may recapitulate molecular switches that operate when neural crest-derived progenitors develop into either melanocytic or glial lineages (55, 56) and may help identify them. We investigated here mechanisms involved in the transcriptional activation of promoters from two myelin genes, Po and MBP. One of the switches that seems to affect both MITF and myelin gene promoters is a profound down-regulation of Pax3 observed following IL6RIL6 treatment. The Pax3 RNA was decreased at 12 h after IL6RIL6, followed by a decrease in the protein (40). Part of this effect may be on stability of Pax3 RNA, because its half-life decreased from 6 to 3 h, 1 day after IL6RIL6 (not shown). Pax3 is an activator of the MITF gene (57), and we have demonstrated that the loss of Pax3 caused by IL6RIL6 is responsible for the silencing of the MITF promoter, because its activity can be restored by ectopic expression of Pax3 (40). As shown here, expression of Pax3 in the same F10.9 cells conversely represses the ability of IL6RIL6 to activate the promoters of the myelin Po and MBP genes. An inhibition of MBP gene induction by Pax3 had been described in SC and in neuroblastoma (20). This is the first report of a similar repression of the Po gene promoter. It is of interest that the down-regulation of Pax3 by the gp130 activator IL6RIL6 is also observed in cultures of embryonic DRG and derived SC, in which Po and MBP gene products are induced (37). Because the decrease in Pax3 is a constant feature of terminal differentiation of myelinating SC (5), this may be an important mechanism by which gp130 signaling enhances myelin gene expression.

Pax3 acts in synergy with Sox10 to activate the MITF promoter, and mutations in any of these three genes cause some form of Waardenburg syndrome with melanocytic deficiencies (45–48). Contrasting with the strong decrease in Pax3 caused by IL6RIL6 in F10.9 cells, the Sox10 protein is rather increased after IL6RIL6 treatment for 2–3 days. It may seem paradoxical that IL6RIL6, which inhibits melanocytic differentiation of the cells, causes an increase in Sox10, a factor needed for melanogenesis (45–48). However, we have shown that, in IL6RIL6-treated F10.9 cells, Sox10 overexpression does not restore MITF and does not synergize with Pax3 as it does in the
untreated F10.9 cells (40). This suggests that the lowering of Pax3 relative to Sox10, and the deregulation of their synergistic interaction, prevent Sox10 from activating MITF and melanogenesis. It may also free Sox10, making it available for interaction, prevent Sox10 from activating MITF and melanogenesis.

For Sp1, a binding site at promoters in HeLa cells (58). This suggests that HeLa cells lack a co-factor for Sox10 or contain an inhibitor. In general, proteins that are positively involved in the response to IL6RIL6. We, therefore, searched for actual mutated sequences see Fig. 5D.

Mutated sites affecting the Po promoter response to IL6RIL6, Sox10, and Krox20

| Mutated sitesa | Po −300/+/45 | Po −300/+/45 with −168/−161 mutationb |
|----------------|--------------|--------------------------------------|
| Wild-type Po −300 | 100 | 100 |
| Sox−10 B + C sites | 13 | 10 |
| CACC−247/−240 | 64 | 72 |
| GGGA−220/−213 | 68 | 60 |
| GTCG−183/−177 | 66 | 71 |
| CACC−165/−161 | 39 | 32 |

a Type of site mutated. For actual mutated sequences see Fig. 5D.

b Activity or fold induction of mutants in percent of the wild-type Po −300/+/45 luciferase reporter construct, in IL6RIL6-treated cells or without IL6RIL6 but with Sox10 or Krox20 co-transfection.

One such element is a long CACC box, which is functionally defined by the −168/−161 mutation inhibiting the Po promoter response to IL6RIL6, as well as to Sox10. Several DNA-protein complexes are formed on a probe containing this CACC box sequence, and some of the complexes are increased following IL6RIL6 treatment whereas other may decrease. We identified a protein that functionally interacts with this CACC box sequence used as cis-acting transcriptional element in a one-hybrid yeast selection system, as transcription factor ZBP-99. The mRNA for ZBP-99 is increased in the F10.9 cells following treatment with IL6RIL6. ZBP-99 had been isolated previously as binding a long CACC box from the gastrin gene promoter and shown to belong to a novel family of proteins defined by an array of four Kruppel-type zinc fingers (51). ZBP-99 was reported to inhibit transcription of the Ornithine decarboxylase gene similarly to its homologous factor ZBP-89/BFCOL1 (51, 61). However, ZBP-89 has also been shown to activate transcription of the p21/waf1 gene through cooperation with histone acetyltransferase p300 in HT29 cells (51). Transfection of the F10.9 cells with ZBP-99 stimulated the activity of the Po promoter, and its action was potentiated when co-transfected with Sox10. Moreover, ZBP-99 was not active on a Po promoter in which Sox10 sites B and C were mutated. These results demonstrate that a protein, acting through the CACC box, stimulates Po promoter activity and mediates a cooperative action of Sox10 with the CACC box element. The increased expression of the Kruppel-type factor ZBP-99 that is observed in the IL6RIL6-treated melanoma cells may be one of the mechanisms activating the Po promoter in these cells. However, complex interactions with other transcription factors and regulatory proteins are likely to be involved in the function of the CACC box and its interplay with the Sox10 sites.

In addition to Pax3, Sox10 and ZBP-99 we examined the role of Egr-2/Krox20 in the activation of the Po gene in F10.9 melanoma cells. Krox20 is required for the onset of myelination during SC development (17), and it controls a large array of SC genes including Po/myelin protein zero (19). Activation of the Po promoter by Krox20 was seen in SC (18) but not in neuroblastoma (16), and synergism of Krox20 and Sox10 was re-
ported on the Connexin-32 promoter (57). In the F10.9 cell system, KroX20 expression activates the Po promoter and exhibits a strong synergism with Sox10. The effect of KroX20 was not related to the −168/−161 CACC box, but we identified other G/C-rich sequences that may mediate its action on the Po promoter. The untreated F10.9 cells already contain KroX20 mRNA, and IL6RL6 did not significantly increase its level, although other types of modulations affecting KroX20 functions are not excluded. In embryonic neuralgic cell cultures from DRG of rat E14 embryos, we did find that IL6RL6 causes a strong increase in KroX20 mRNA and protein, in correlation with the induction of MBP and Po mRNAs.

There is growing evidence for a major role of the IL-6 cytokine family in the synthesis of myelin. In vivo, the inactivation of the IL-6 family gp130 receptor in newborn mice was shown to impair nerve myelinization (38). Using the potent gp130 activator IL6RL6, we reported in vivo stimulation of myelinogenesis in regenerating sciatic nerves (37) and in vitro activation of myelin gene expression in premyelinating Schwann cells from embryonic DRG (36, 37). The present melanoma cell system provides a new model to investigate the multiple mechanisms that activate myelin gene transcription in response to the IL-6-type stimulus.

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