Mammalian Achaete Scute Homolog 2 Is Expressed in the Adult Sciatic Nerve and Regulates the Expression of Krox24, Mob-1, CXCR4, and p57kip2 in Schwann Cells

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The molecular control mechanisms and regulatory molecules involved in nerve repair are not yet well known. Schwann cells have been attributed an important role in peripheral nerve regeneration; therefore, attention has been drawn to regulatory factors expressed by these glial cells. Here, we demonstrate that Mash2, a basic helix–loop–helix (bHLH) transcription factor previously shown to be crucial for placenta development, is expressed by Schwann cells of adult peripheral nerves. We observed that this gene is downregulated after nerve lesion and, using cDNA array hybridization technology, we could demonstrate that Mash2 is a regulator of Krox24, Mob-1, and CXCR4 expression in cultured Schwann cells. In addition, we provide strong evidence that Mash2 is a negative regulator of Schwann cell proliferation. Mash2 represents a first candidate for the missing class B bHLH proteins in peripheral nerves.

Key words: basic helix–loop–helix; Schwann cell; cDNA array hybridization; Wallerian degeneration; proliferation; cytokine

The adult peripheral nervous system retains the capacity to regenerate after injury, and it has been shown that this is a consequence of molecular interactions occurring between axotomized neurons, Schwann cells, and phagocytosing macrophages. After injury, the axons distal to the lesion site degenerate, myelin sheaths are broken down, and debris is removed by both Schwann cells and macrophages. This so called Wallerian degeneration is essential for successful nerve regeneration, and during this period, Schwann cells undergo dedifferentiation and proliferate to generate a guidance substrate for growing axons (Fawcett and Keynes, 1990).

During the past decade, a large number of differentially expressed genes have been identified that are thought to be involved in peripheral nervous system repair. However, little information exists regarding the identity of transcriptional regulators that direct the coordinated expression of target genes during the distinct stages of nerve degeneration and regeneration (Gillen et al., 1995; Küry et al., 2001). Conversely, Schwann cell differentiation has been shown to depend primarily on the expression of the transcription factors Pax3, Sox10, Oct6 (SCIP or Tst-1), and Krox20 (Scherer et al., 1994; Topilko et al., 1994; Kioussi et al., 1995; Bermingham et al., 1996; Jaegle et al., 1996; Britsch et al., 2001).

Until now, no cell-specific (class B) basic helix–loop–helix (bHLH) proteins could be detected in Schwann cells, but several lines of evidence proposed a role of these transcription factors in peripheral nerves, such as the observation that class A transcripts of the bHLH genes REB and E2A occur in mature Schwann cells and the fact that bHLH transcription factors act as A/B heterodimers. In addition, the presence of E-box binding sites in promoters of several Schwann cell genes also argues for a function of bHLH proteins in this glial lineage (Stewart et al., 1997). In addition, non-DNA-binding Id helix–loop–helix (HLH) proteins known to interact with bHLH transcription factors and to interfere with their transcriptional activities were found to be expressed throughout development and in mature Schwann cells (Stewart et al., 1997; Thatikunta et al., 1999).

Mammalian achaete scute homolog (Mash) genes were initially discovered in a neural crest-derived cell line (Johnson et al., 1990). Whereas Mash1 was shown to be involved in the determination and differentiation of neurons (for review, see Guillemot, 1999), Mash2 has thus far been implicated only in trophoblast development during early embryogenesis, with gene inactivation resulting in embryonic lethality (Guillemot et al., 1994). Here, we demonstrate that the Mash2 gene is expressed by Schwann cells of the adult peripheral nerve. This transcription factor represents a first candidate for the missing class B bHLH proteins, and we found that after sciatic nerve crush, Mash2 expression was decreased. By applying cDNA array hybridization technology, we identified Schwann cell-specific downstream target genes of this transcriptional regulator. We provide strong evidence that Mash2 is involved in both Schwann cell differentiation and the control of proliferation occurring in the injured nerve.

MATERIALS AND METHODS

Antibody generation, immunohistochemistry, and in situ *hybridization*. Two different rabbit polyclonal anti-Mash2 antibodies (rabbit 1 and rabbit 2) were produced using the peptide MESSGKMESGAGQQPQPPQP corresponding to the 20 N-terminal amino acids of the rat Mash2 protein. Both immune sera were affinity-purified using N-terminal Mash2 peptide-coupled Sepharose 4B column chromatography. SDS-PAGE and Western blotting were performed according to Laemmli (1970) and Towbin et al. (1979), respectively, using a horseradish peroxidase-conjugated goat anti-rabbit antibody (Southern Biotechnology, Alabaster, AL) and an ECL Western blot detection system (Amersham Biosciences, Arlington Heights, IL). Immunostainings of cryostat sections

Received December 19, 2001; revised March 27, 2002; accepted May 29, 2002.

This work was supported by Deutsche Forschungsgemeinschaft Grant Mu 630/8-1. We thank B. Ziegler for preparation of rat Schwann cells, F. Bosse and M. Czardybon for supplying the CXCR4 cDNA, D. Abankwa for help with gene expression array analysis, and T. Herdegen for the kind gift of anti-Krox24 antibody.

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using affinity-purified Mash2 antibodies (rabbit 1 and rabbit 2, diluted 1:50) were performed overnight at 4°C in PBS containing 3% normal goat serum. Rabbit anti-S100 (Sigma, St. Louis, MO), rabbit anti-low-affinity nerve growth factor receptor (LNGFR) (Chemicon, Temecula, CA), rabbit anti-Krox24 (Herdegen et al., 1991), rabbit anti-egr2 (Krox20; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-p57kip2 (Acris, Hiddenhausen, Germany) antibodies were used at dilutions of 1:200, 1:50, 1:4000, 1:50, and 1:20, respectively, using the same incubation buffer. Horseradish peroxidase-, Cy3-, and Alexa green-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA; Molecular Probes, Eugene, OR) were used for signal visualization. A Sybr Green nucleic acid staining kit was purchased from Molecular Probes. Production of digoxigenin (DIG)-labeled cRNA probes was performed by *in vitro* transcription of Mob-1 and CXCR4 cDNA templates (472 and 772 base pairs, respectively) according to the instructions of the supplier (Roche Products, Hertfordshire, UK). *In situ* hybridization of paraffin sections was performed at 55°C overnight according to Angerer et al. (1987). Visualization using the alkaline phosphatase-coupled anti-DIG antibody was performed according to the protocol of the supplier (Roche).

Animal surgery. Adult Wistar rats were anesthetized with chloral hydrate (350 mg/kg body weight) by intraperitoneal administration. Sciatic nerves were crushed using fine jeweler's forceps according to Müller et al. (1986). After nerve transection, both nerve ends were tied off to prevent axons from growing into the distal stump. The rats were killed by cervical dislocation 2–28 d after nerve injury, and the tissue distal to the site of lesion was dissected with care taken that the lesion zone itself was omitted. Six to eight sciatic nerves were pooled for each time point. All animal experiments were performed according to the guidelines of the German Animal Rights Law.

RNA preparation. Total RNA derived from adult rat sciatic nerves was prepared according to the method described by Chomczynski and Sacchi (1987). Total RNA derived from cultured Schwann cells was prepared using the RNeasy procedure according to the protocols of the supplier (Qiagen, Hilden, Germany). Before additional use, total RNA was DNase I-digested according to the instructions of the supplier (Roche).

Transfection and Schwann cell sorting. Rat Schwann cells were prepared according to Brockes et al. (1979) and grown at a density of 10,000 cells/cm^2 on poly-D-lysine (Sigma) in DMEM (Invitrogen, San Diego, CA) in the presence of 10% fetal calf serum (FCS; Invitrogen) and 2 μM forskolin (Sigma). Fugene 6 (Roche) mediated cotransfection of either pMash2–internal ribosome entry site 2 (IRES2)–enhanced green fluorescent protein (EGFP), an 882 base pair fragment corresponding to the entire coding region of the rat Mash2 cDNA cloned in the pIRES2–EGFP vector (Clontech, Cambridge, UK; see below for oligonucleotides used for amplification) or pIRES2–EGFP, together with a CD14 expression vector (pMACS-14.1, Miltenyi Biotec, Bergisch-Gladbach, Germany), was performed at a ratio of 5:1. Forty-eight hours after transfection, cells were harvested and incubated with an anti-CD14 antibody coupled to magnetic microbeads. Cells were then sorted using a magnetic column according to the manual (Miltenyi Biotec). Using this procedure, we received ~70% pure populations of transiently transfected Schwann cells. Bromodeoxyuridine (BrdU) incorporation during DNA replication was used to determine Schwann cell proliferation (Gratzner, 1982). BrdU and anti-BrdU antibodies were used according to the protocols of the supplier (Roche).

Expression array analysis. The experiments were performed on rat 1.2 Atlas array filters (Clontech). cDNA probe synthesis using 5 μg of DNA-free total RNA, filter hybridization, and washings were all performed according to the instructions of the supplier. Hybridized filters were scanned (BAS reader, Fujifilm; Medical Systems, Stamford, CT) and analyzed using TINA software (Raytest, Straubenhardt, Germany). See Results section for a detailed description of the analysis procedure.

Reverse transcription and PCR. Reverse transcription (RT) of total RNA was performed using the Superscript II enzyme (Invitrogen) and dT_{19}(A/C/G) oligonucleotide primers. PCR amplification of the rat Mash2 cDNA was performed using the Amplitaq polymerase (Applied Biosystems, Foster City, CA) with oligonucleotides at a concentration of 0.33 pmol/μl in a buffer consisting of 1.5 mM $MgCl_2$, 5% DMSO, and 1 mM dNTPs. The amplification profile was 30 sec at 94°C, 1 min at 55°C, and 1 min at 72°C. The sequence of oligonucleotides was Mash2-forward TGGAATCGCACTTTAACTGG, Mash2-reverse CTAGACAGCATGGGTAAGGC. DNA sequencing was performed on a 310 Genetic Analyser using the reagents of the supplier (Applied Biosystems).

Real-time quantitative PCR was performed on a 5700 GeneAmp (Applied Biosystems) using the Sybr Green assay according to the protocols of the supplier. The sequence of oligonucleotides was Mash2-forward AACTTTCCAACCTGGCCAAGGT, Mash2-reverse AG-CCAGGCATCTTGCCC, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-forward GAACGGGAAGCTCACTGGC, GAPDH-reverse GCATGTCAGATCCACAACGG, Krox24-forward CCCTGTTGAGTCCTGCGATC, Krox24-reverse GGCGTGTAAGCTCATCCGAG, Mob-1-forward AGACCGATGGACAGCAGAGAG, Mob-1-reverse ACACTGGGTAAAGGGAGGTGG, CXCR4-forward CGTCGTGCACAAGTGGATCT, CXCR4-reverse CAGTGGAAGAAGGCGAGGG, p21cip1-forward TTCTTCTGCTGTGGGTCAGGA, p21cip1-reverse AAGGCTAAGGCAGAAGATGGG, p27kip1-forward GCGTTTCGCTTTTGTTTGGT, p27kip1-reverse ACGTTTGACATCTTCCTCCCC, p57kip2-forward CAGGACGAGAATCAGGAGCTGA, p57kip2-reverse TTGGCGAAGAAGTCGTTCG, Krox20-forward TTTTTCCATCTCCGTGCCA, and Krox20-reverse GAACGGCTTTCGATCAGGG.

RESULTS

Mash2 is expressed by Schwann cells of the adult sciatic nerve

Using RT-PCR to selectively amplify cDNA fragments of regulatory genes, we found that the bHLH transcription factor Mash2 is expressed in adult rat sciatic nerves and cultured rat Schwann cells (data not shown). We then used quantitative real-time RT-PCR, using the expression of GAPDH as an internal standard, and could demonstrate that after both nerve crush and nerve transection, expression of Mash2 is transiently downregulated (Fig. 1*A*). This provides evidence that decreased Mash2 activity is linked to Wallerian degeneration and not directly to regeneration events. To detect Mash2 protein, we generated two different rabbit polyclonal antisera directed against the N-terminus of the rat protein. Affinity purification using this N-terminal Mash2 peptide resulted in two polyclonal anti-Mash2 antibodies (rabbit 1 and rabbit 2), which we subsequently tested by means of immunocytochemistry on Mash2-transfected Schwann cells (coexpression of EGFP served as control; data not shown). Western blot analysis then revealed that both antibodies recognized a band of ~38 kDa in extracts derived from both embryonic day 15.5 (E15.5) rat placenta (*lane 1*) and cultured rat Schwann cells (*lane 2*), which corresponded to a band seen in Mash2-transfected cos-7 cells (*lane 4*) but was absent in cos-7 cells transfected with the empty expression vector (*lane 3*), as shown for the rabbit 1 anti-Mash2 antibody in Figure 1*B*. Immunohistochemistry on cryostat sections of adult rat sciatic nerves revealed that both purified antibodies produced very similar staining patterns, resembling the distribution of Schwann cells within adult peripheral nerves (Fig. 1*C*,*C′*). Additional staining in the perineurium was found to be nonspecific and a consequence of the staining procedure and could also be observed in control stainings in which the first antibody was omitted (Fig. 1*D*). However, we also found Mash2 being expressed by endothelial cells (*open arrow* in Fig. 1*C′*). This signal appeared to be specific and was not affected by the nerve lesion, because we also detected it in blood vessels of injured nerves stumps (data not shown). To prove that the anti-Mash2 antibodies selectively labeled Schwann cells, we applied anti-S100 antibodies (Haimoto et al., 1987) to adjacent sections and found that the two signals overlapped (Fig. 1*E*,*F*). Direct comparison of the Mash2 signals with Sybr Green-stained Schwann-cell nuclei was performed using confocal microscopy and demonstrated that most of the Mash2 protein was located in the nucleus, but with a smaller fraction residing outside the nucleus (Fig. 1*G–I*). Similarly, Mash2 immunocytochemistry on cultured Schwann cells revealed a strong nuclear signal and some staining outside the nucleus (Fig. 1*J*).

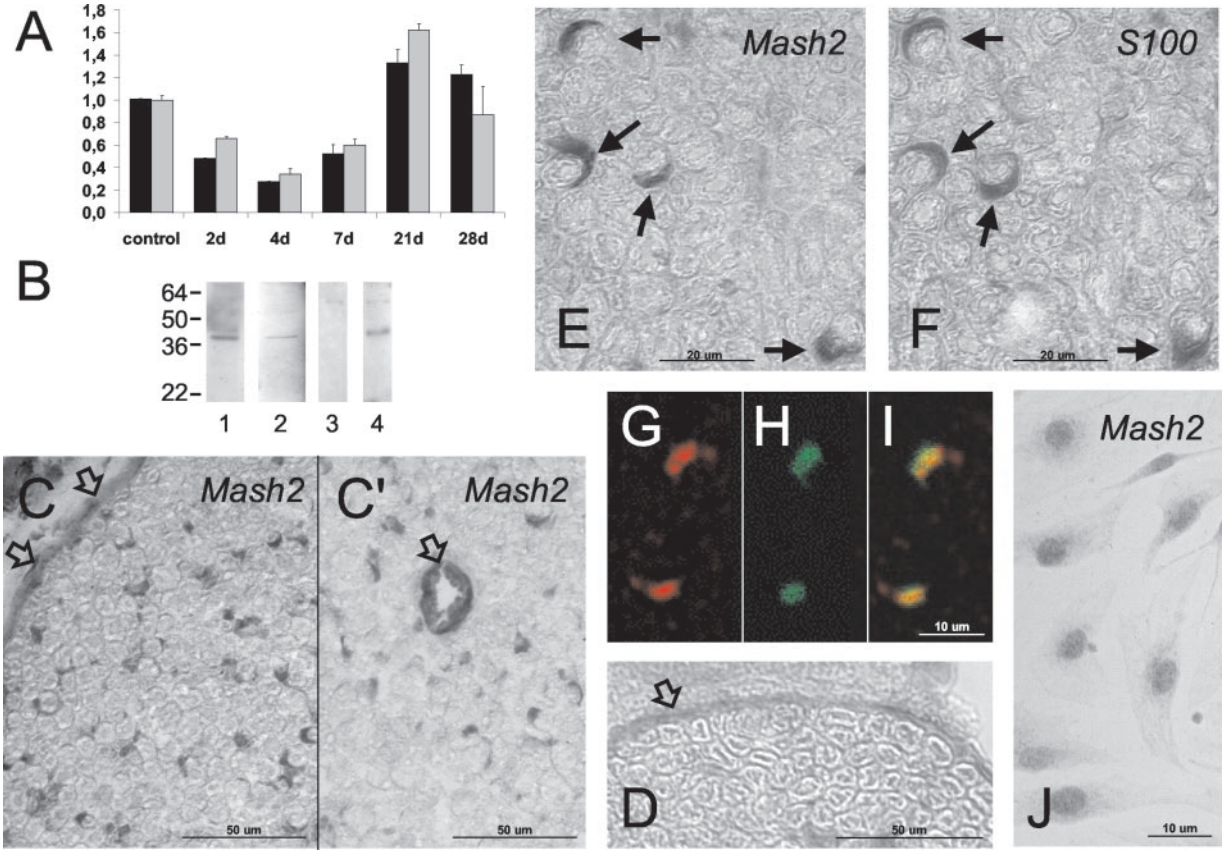

Figure 1. Mash2 is expressed in Schwann cells of the adult sciatic nerve. *A*, Quantitative RT-PCR analysis revealed a transient downregulation of Mash2 expression after both nerve crush (*black columns*) and nerve transection (*gray columns*; *2–28d*, days after nerve injury). *B,* Western blot analysis using the rabbit 1 anti-Mash2 antibody on E15.5 placenta (*lane 1*, 15 μg of protein), Schwann cell (*lane 2*, 40 μg of protein), control, and Mash2 transfected cos-7 cell extracts (*lanes 3* and *4*, 5 μg of protein each) demonstrated that a 38 kDa band corresponding to the rat Mash2 protein occurred in placenta and in Schwann cells. *C*, *C′*, Immunostainings of adult rat sciatic nerve cross sections using rabbit 1 anti-Mash2 (*C*) and rabbit 2 anti-Mash2 (*C′*) antibodies. *D*, Control immunostaining in which anti-Mash2 antibodies were omitted. *E*, Rabbit 1 anti-Mash2 immunostaining; *F,* anti-S100 immunostaining of a section adjacent to the one shown in *E* demonstrating that Mash2 is expressed by S100-positive Schwann cells. *G–I,* Rabbit 1 anti-Mash2 immunostaining (*G*), Sybr Green nuclear staining of the same section (*H*), and the merged pictures as revealed by confocal microscopy (*I*). *J,* Rabbit 1 anti-Mash2 immunocytochemistry of cultured rat Schwann cells. *Arrows* mark double-labeled Schwann cells, and *open arrows* in *C* and *D* mark the perineurium and Mash2 expression in endothelial cells (*C′*), respectively. Scale bars: *C, C′, D*, 50 μm; *E, F*, 20 μm; *G–J*, 10 μm.

Gene expression arrays reveal Mash2 downstream target genes in Schwann cells

As a transcription factor, the primary role of Mash2 is to regulate gene activities, and identifying those target genes will provide important information about the function of Mash2 in the peripheral nerve. We therefore aimed to identify which genes were affected in Schwann cells overexpressing this regulatory factor. Because three different attempts to generate stably transfected Schwann cells failed (data not shown), we developed a transient expression assay.

Schwann cells were transfected with a Mash2–EGFP expression vector (pMash2–IRES2–EGFP) or, in parallel, with an EGFP control expression vector (pIRES2–EGFP). After 48 hr in culture, transfected cells were sorted and lysed (see Materials and Methods). The initial transfection efficiency was up to 20%, and after the sorting procedure, ~70% of the Schwann cells were EGFP-positive. Subsequently, DNA-free total RNA was prepared and reverse transcribed to generate ^{32}P-labeled cDNA pool probes. Those probes were used to hybridize gene expression arrays (Clontech rat 1.2 Atlas array filters) to detect differences in the expression levels among the 1176 dotted gene fragments. Six independent experiments were performed in which Mash2–EGFP-overexpressing cells were directly compared with EGFP-overexpressing control cells. Hybridized array filters were scanned, and the signal intensities were quantified. Each hybridization signal was scored using a threshold value that we defined as the background signal plus two SDs. This threshold value was used as a quality criterion and helped to decide whether a given signal was reliable and could be discriminated unambiguously from background radiation. Then, each signal was compared with the expression level of five reference genes, such as GAPDH, ornithine decarboxylase (ODC), β-tubulin, and α-actin, as well as a virtual reference gene, which was the calculated average signal intensity. In addition, we defined a regulation threshold, which means that only those genes were scored that had expression levels upregulated or downregulated at least threefold. Finally, a visual inspection of regulated gene spots was performed. Applying these criteria, we found that of the 1176 dotted genes, the expression levels of 711 genes could be quantified and analyzed. Of those, we detected three genes whose expression was changed specifically in Mash2-overexpressing Schwann cells. In six of six experiments, we found that the transcription factor gene Krox24 (Topilko et al., 1997) was suppressed at elevated Mash2 levels (Fig. 2, compare *A, A′*). In five of six experiments, we observed

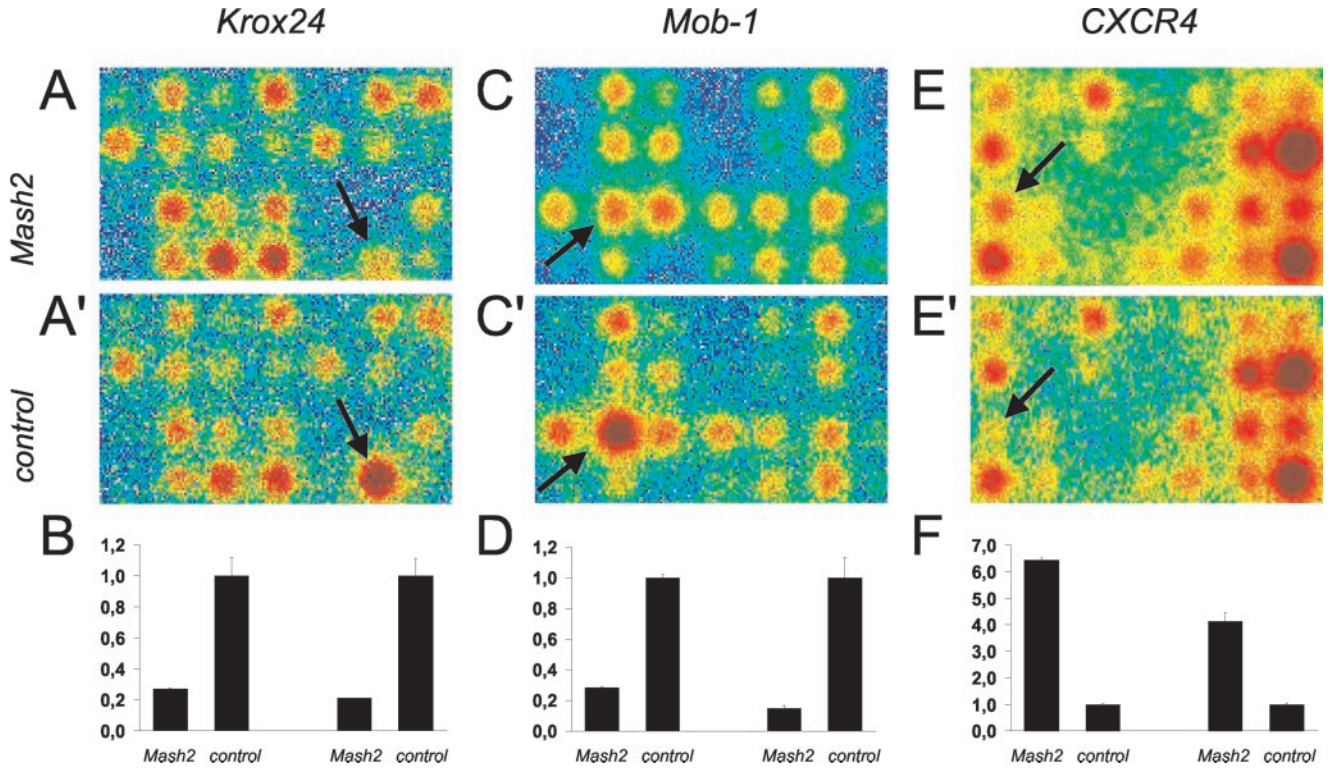

Figure 2. Krox24, Mob-1, and CXCR4 are downstream target genes of Mash2 in cultured Schwann cells. *A–E′*, Gene expression array of Schwann cells transiently transfected with pMash2–IRES2–EGFP (*A, C,* and *E*) or Schwann cells transfected with the control vector pIRES2–EGFP (*A′, C′,* and *E′*). Krox24 (*arrows* in *A* and *A′*) and Mob-1 (*arrows* in *C* and *C′*) are downregulated by Mash2 and the CXCR4 gene is upregulated (*arrows* in *E* and *E′*). *B, D, F,* Quantitative RT-PCR analysis confirmed that Mash2 is a downregulator of Krox24 (*B*) and Mob-1 (*D*) and upregulates CXCR4 (*F*). For each gene, cDNA quantification of two separate experiments is shown. GAPDH expression was used as reference, and data are mean values ± SD. *Mash2*, pMash2–IRES2–EGFP; *control*, pIRES2–EGFP-transfected cells.

that expression levels of the chemokine Mob-1–IP10–CRG-2 (Liang et al., 1994) were decreased (Fig. 2, compare *C, C′*), whereas the chemokine receptor gene CXCR4 (Nagasawa et al., 1996) was upregulated by Mash2 (Fig. 2, compare *E, E′*). Interestingly, none of the other known Schwann cell genes present on the array filter, such as P0, PMP22, LNGFR, erbB2, erbB3, and integrin β4 (all reviewed in Jessen and Mirsky, 1999), were found to be affected by Mash2 (data not shown), indicating that the Mash2-induced changes in gene expression were specific but also suggesting that the expression of myelin genes is not under the control of Mash2.

We then used quantitative RT-PCR to verify that these three genes were, indeed, affected by Mash2. We used GAPDH (Fig. 2) and ODC (data not shown) as reference genes. As shown for two separate experiments (Fig. 2*B,D,F*), Krox24 and Mob-1 were confirmed to be suppressed, and CXCR4 was shown to be induced by Mash2. Considering that our sorting procedure did not result in pure populations of transiently transfected cells, the actual differences in transcript levels of these three genes may even be higher.

Cellular colocalization of Mash2, Krox24, Mob-1, and CXCR4

After the observation that in cultured Schwann cells, Krox24 expression is suppressed by Mash2 (Fig. 2), we wanted to discover whether in the adult rat sciatic nerve, these two transcription factors are coexpressed or whether they are mutually exclusive. We performed immunohistochemistry on adjacent adult nerve sections using rabbit polyclonal antibodies directed to Krox24 (Herdegen et al., 1991) and Mash2. We noticed that the number of Mash2-expressing cells exceeded the number of Krox24-positive cells (Fig. 3*A,B*), which is in agreement with the distribution of Krox24 in the adult peripheral nerve, as published by Topilko et al. (1997). A few cells, however, were found to express both transcription factors (Fig. 3*A′,B′*). Because these authors have proposed that these Krox24-positive cells are nonmyelinating Schwann cells, we performed immunohistochemistry on adjacent nerve sections using antibodies directed against LNGFR (Jessen et al., 1990) as a marker for nonmyelinating Schwann cells and Mash2. As shown in Figure 3, *G* and *H*, no cells expressing both proteins could be detected. Conversely, we also performed additional parallel stainings using rabbit anti-Krox20 antibodies and found numerous Schwann cell nuclei that expressed both proteins, Krox20 and Mash2 (Fig. 3*C,D*), thus demonstrating that myelinating Schwann cells express the Mash2 gene *in vivo*. Applying real-time quantitative RT-PCR, the expression profiles of Krox20 after nerve crush and nerve transection were established (Fig. 3*E*). This demonstrated that although in the crushed nerve Mash2 and Krox20 displayed almost identical expression profiles, in the transected nerve Krox20 remained downregulated, whereas Mash2 was again upregulated beyond postlesion day 7 (Fig. 1*A*). This provides additional evidence that Mash2 is not part of the myelination program as such but that its downregulation is linked to molecular and cellular events occurring during Wallerian degeneration. We then wanted to know whether Mash2 could also affect the expression of this gene and determined levels of Krox20 expression in transiently transfected Schwann cells. No differ-

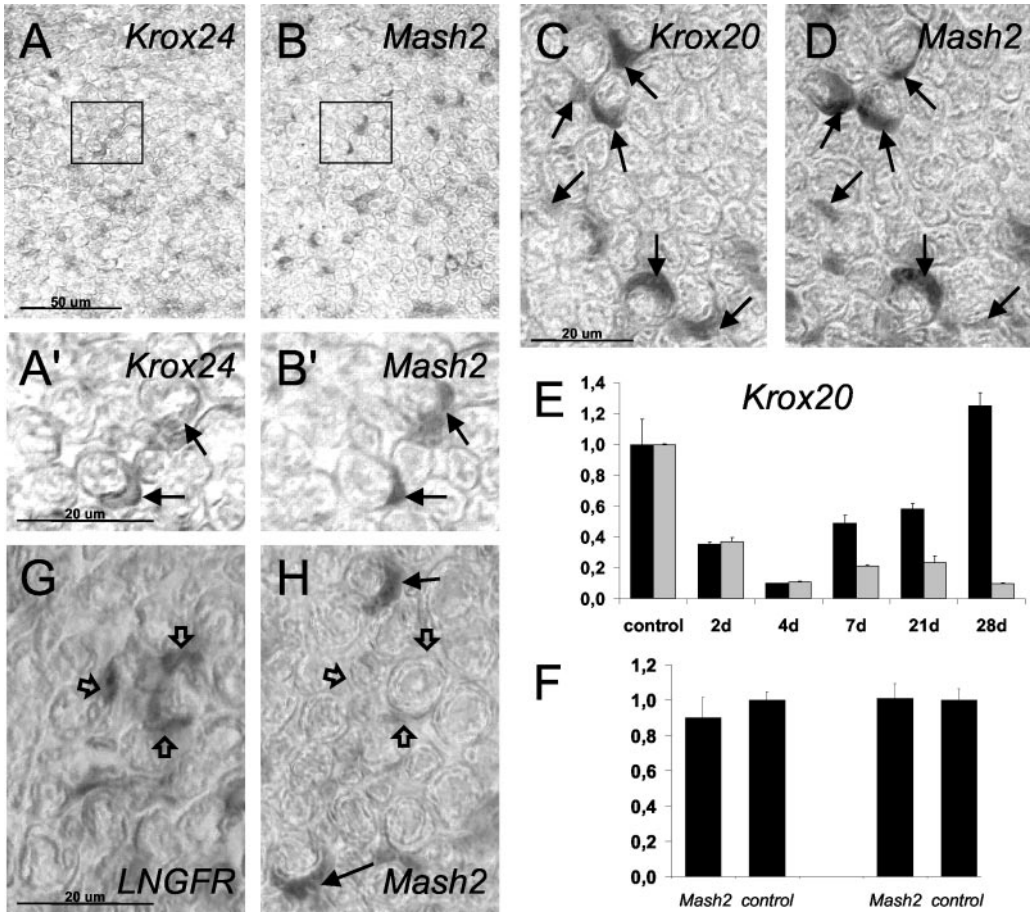

Figure 3. Mash2 is expressed in myelinating Schwann cells. *A*, Immunostaining of an adult rat sciatic nerve cross section using anti-Krox24 antibodies. *B*, Same field on a section adjacent to the one shown in *A* stained with the rabbit 1 anti-Mash2 antibody. *A′, B′,* Enlargements of *boxed areas* in *A* and *B*, respectively, demonstrating that Krox24 and Mash2 are expressed in some Schwann cells (*arrows*). *C,* Immunostaining of an adult rat sciatic nerve section using anti-Krox20 antibodies. *D*, Same field on an adjacent section stained with the rabbit 1 anti-Mash2 antibody revealing many myelinating Schwann cells expressing Mash2 (*arrows*). *E,* Quantitative RT-PCR analysis demonstrating a transient downregulation of Krox20 expression after nerve crush (*black columns*) and a continued downregulation on nerve transection (*gray columns*; 2–28 d after nerve injury). *F,* Quantitative RT-PCR analysis indicates that Mash2 is not a regulator of Krox20 expression. cDNA quantification of two separate experiments is shown; GAPDH expression was used as reference, and data are mean values ± SD. *Mash2*, pMash2–IRES2–EGFP; *control*, pIRES2–EGFP-transfected cells. *G*, Immunostaining of an adult rat sciatic nerve section using anti-LNGFR antibodies. *H*, Section adjacent to the one shown in *G*, demonstrating that nonmyelinating Schwann cells do not express Mash2. *Arrows* mark Mash2-labeled Schwann-cell nuclei; *open arrows* mark LNGFR signals. Scale bars: *A*, 50 μm; *A′, C, G*, 20 μm.

ences in Krox20 transcript levels could be detected (Fig. 3*F*), indicating that the effect on Krox24 expression was highly specific.

A combination of *in situ* hybridization and immunofluorescence was used to perform colocalization studies for CXCR4 and Mob-1. Adult rat sciatic nerve sections were hybridized with digoxigenin-labeled antisense cRNA probes and subsequently subjected to immunofluorescence using anti-Mash2 antibodies (Fig. 4). This demonstrated that not only in cultured Schwann cells but also in the sciatic nerve, both genes CXCR4 and Mob-1 are coexpressed with their transcriptional regulator Mash2. No specific signals were detected using the sense cRNA probes (Fig. 4*E,F*).

Mash2 acts as a regulator of Schwann cell proliferation

Depending on the culture conditions, Schwann cells can adopt different morphologies and proliferation rates. In the presence of high serum and the cAMP agonist forskolin, rat Schwann cells are flat and nonpolar and proliferate quickly. If forskolin is withdrawn and the serum content is reduced to the minimum necessary to ensure survival, these cells become elongated and bipolar or tripolar and stop proliferating. We prepared cDNA from rat Schwann cells that were cultured in DMEM for 48 hr under four different conditions: 10% FCS and 2 μM forskolin, 10% FCS only, 0.5% FCS and 2 μM forskolin, and 0.5% FCS only, and performed real-time quantitative RT-PCR to determine relative levels of gene expression using GAPDH as reference gene. Schwann cells were cultured under subconfluent conditions, and we observed that Mash2 expression gradually increased as mitogenic stimuli were reduced (Fig. 5*A*), suggesting that Schwann cell proliferation and Mash2 expression are linked. To reveal whether increased Mash2 levels could directly influence proliferation, we performed S-phase labeling using BrdU. Schwann cells were transfected with the Mash2 expression vector (pMash2–IRES2–EGFP) and the EGFP control expression vector (pIRES2–EGFP), sorted, and subjected to an 8 hr BrdU pulse. This revealed that although fully stimulated by serum and forskolin, increased Mash2 expression significantly reduced the number of proliferating Schwann cells (Fig. 5*B*), thus providing evidence that the capacity of the cell to undergo mitosis is directly linked to Mash2 expression. The control of the mammalian cell cycle is

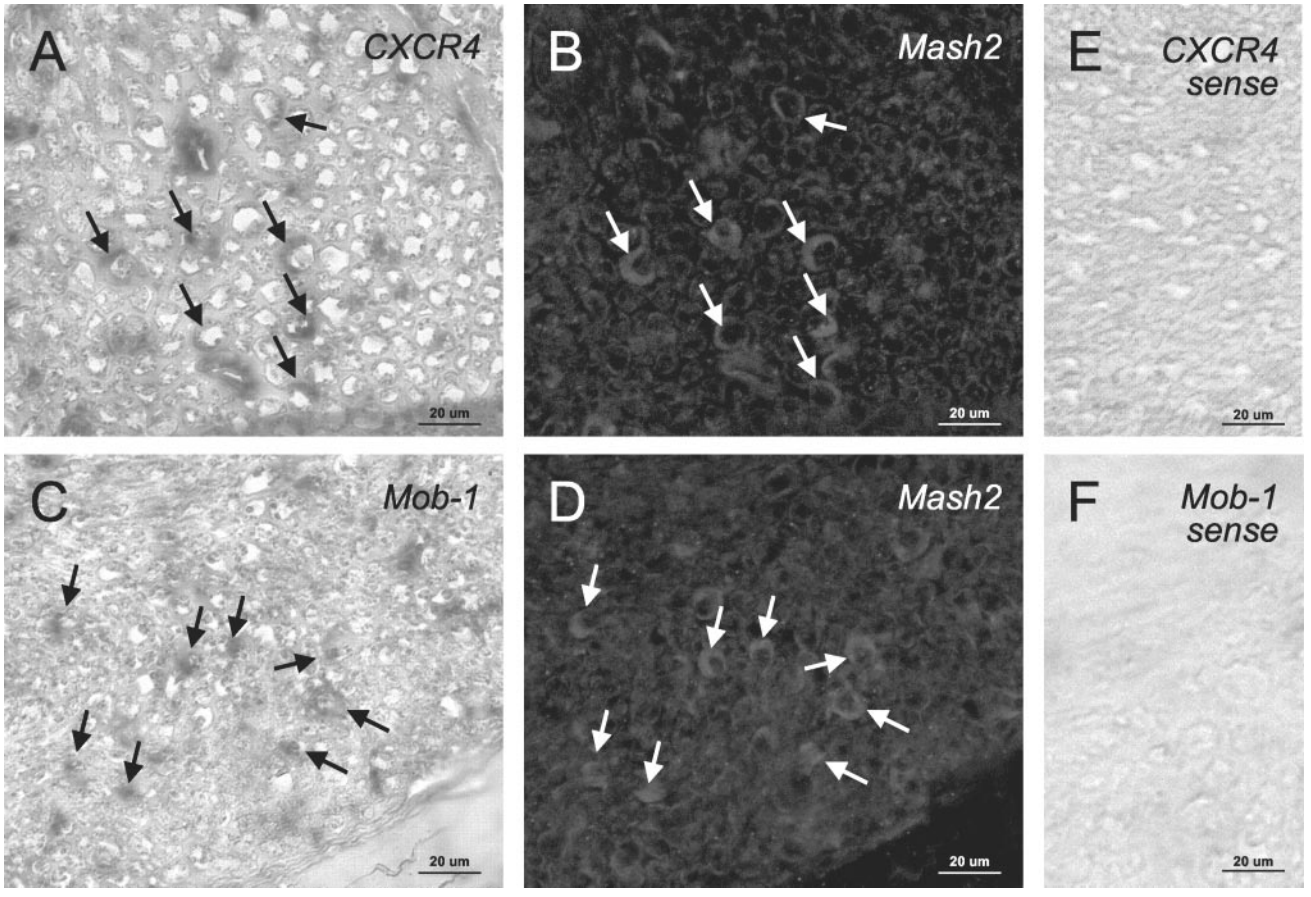

Figure 4. CXCR4 and Mob-1 are expressed in Mash2-positive Schwann cells. *A*, *In situ* hybridization of an adult rat sciatic nerve section using an antisense CXCR4 cRNA probe. *B*, Mash2 immunofluorescence (rabbit 1 anti-Mash2 antibody) on the same section, demonstrating overlapping expression patterns. *C*, Sciatic nerve section hybridized with an antisense Mob-1 cRNA probe. *D*, Same section immunostained for Mash2, revealing coexpression of both genes. *E, F*, Sense cRNA probe hybridization as controls. *Arrows* mark double-labeled Schwann cells. Scale bars, 20 μm.

dependent primarily on the expression and activity of protein complexes consisting of cyclins and cyclin-dependent kinases (CDKs). Conversely, CDK inhibitors, such as p21cip1, p27kip1, and p57kip2, control the G_1–S transition and act as antiproliferative agents (for review, see Sherr and Roberts, 1999). Using real-time quantitative RT-PCR, we could demonstrate that overexpression of Mash2 in Schwann cells results in a strong induction of p57kip2 transcript levels (Lee et al., 1995; Matsuoka et al., 1995), whereas expression of p21cip1 and p27kip1 remained unaffected (shown for two separate experiments each in Fig. 5*C*). Double immunofluorescence on adult sciatic nerve sections then demonstrated the coexpression of p57kip2 and Mash2 proteins in Schwann cell nuclei (Fig. 5*D*).

Gene expression profiles after sciatic nerve injury

We used quantitative RT-PCR (using GAPDH as reference gene) to compare the expression levels of Mash2 with its target genes after sciatic nerve crush. We measured transcript levels of Krox24, Mob-1, CXCR4, and p57kip2 within the same RNA preparations and found that Krox24 and Mob-1 were induced after sciatic nerve crush, being highest at day 7 (Fig. 6*A,B*). This is in agreement with previous data published by Topilko et al. (1997) for Krox24 and with a role for Mash2 as a transcriptional suppressor of Krox24 and Mob-1. However, we also observed that both gene expression profiles experienced strong oscillations, with transient minima at postlesion days 4 and 21. Currently, this oscillatory expression behavior of Krox24 and Mob-1 cannot be explained, but it indicates that apart from Mash2, other transcriptional regulators influence the expression of these genes. p57kip2 and CXCR4 expression, conversely, were found to decrease 2 d after the nerve was injured and to increase again thereafter (Fig. 6*C,D*), which supports our findings that both genes are induced by Mash2. However, for CXCR4, no steady increase could be observed, because 3 weeks after the lesion, expression levels were found to be low again, suggesting additional transcriptional regulator(s) with regeneration-associated activity acting on the expression of this receptor gene.

DISCUSSION

Here, we describe the expression of the first cell-specific bHLH factor found in Schwann cells. During Wallerian degeneration, this gene becomes transiently downregulated, suggesting that high Mash2 activity is linked to Schwann cell quiescence and maturation, an observation that is in agreement with the lesion-induced expression of Id transcriptional antagonists (Stewart et al., 1997). Although it has been proposed that bHLH proteins control myelin gene expression (Thatikunta et al., 1999), we could demonstrate that Mash2 does not affect PMP22 and P0 transcription, which suggests that additional bHLH proteins must exist in Schwann cells. This is also supported by the observation that in contrast to myelin genes or the myelination factor Krox20 (Fig.

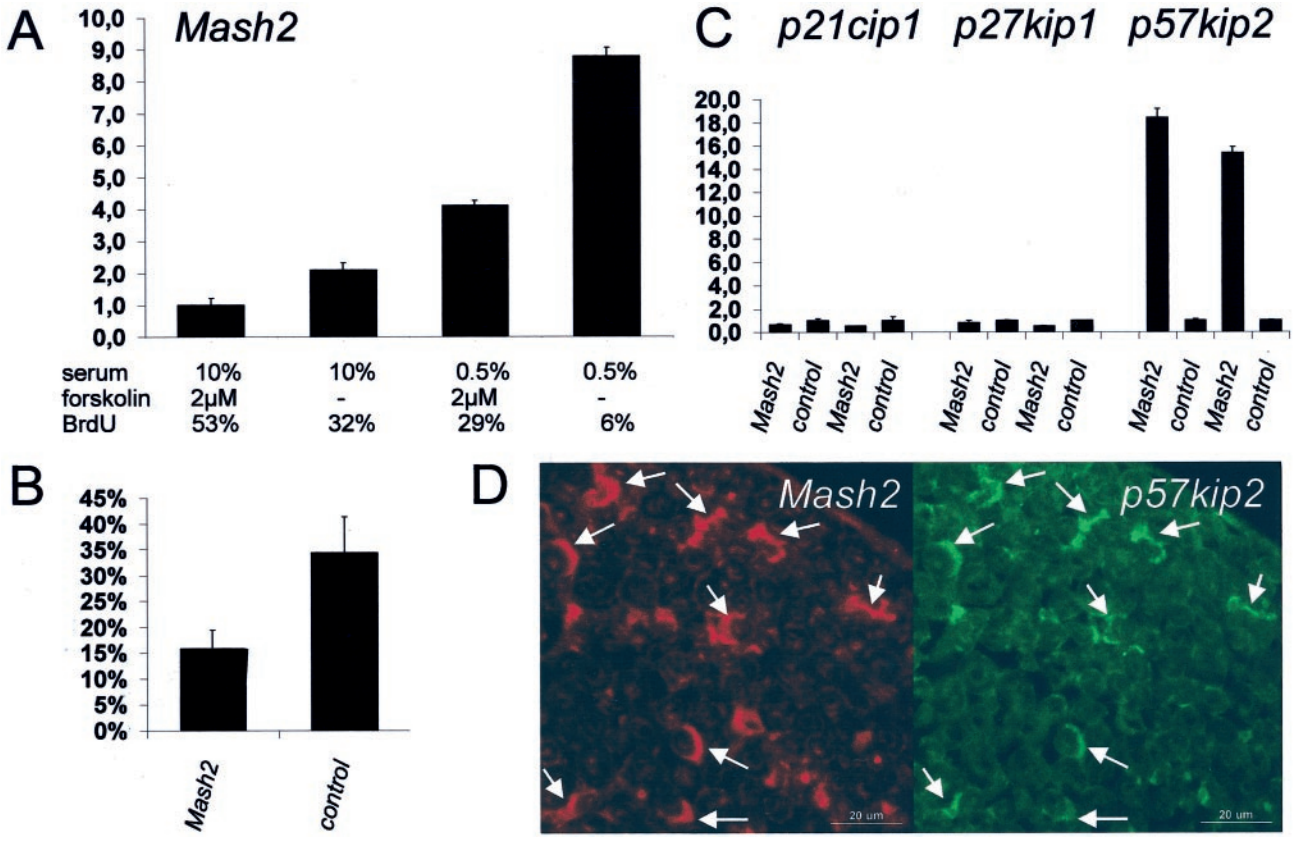

Figure 5. Mash2 regulates Schwann cell proliferation. Quantitative RT-PCR analysis of Mash2 expression levels in Schwann cells grown under different conditions was performed. *A*, Stepwise withdrawal of serum and forskolin resulted in a strong increase of Mash2 expression, whereas the percentage of BrdU-labeled nuclei decreased (16 hr pulse). One representative experiment of five is shown; GAPDH expression was used as reference, and data are mean values ± SD. *B,* Forced expression of Mash2 results in a reduction of proliferating Schwann cells, as revealed by the percentage of BrdU-labeled nuclei. *control*, pIRES2–EGFP-transfected Schwann cells. One representative experiment out of four is shown. *C*, Quantitative RT-PCR analysis of cyclin-dependent kinase inhibitor genes in Mash2-overexpressing Schwann cells, demonstrating that p57kip2 is a specific target of Mash2. For each gene, the result of two separate experiments is shown. GAPDH expression was used as reference, and data are mean values ± SD. *D,* Double immunohistofluorescence of an adult sciatic nerve cross section using rabbit 1 anti-Mash2 antibody (*left*) and mouse anti-p57kip2 antibody (*right*), demonstrating that these two proteins are coexpressed. *Arrows* indicate double-labeled Schwann cells. Scale bars, 20 µm.

3*E*), the expression of Mash2 after nerve transection is induced again (Fig. 1*A*). Nevertheless, it appears that in lesion-induced dedifferentiation and redifferentiation of Schwann cells, bHLH-mediated transcriptional activities must be reduced, which is in agreement with Mash2 acting as a negative regulator of Schwann cell proliferation. This might also explain why it was not possible to generate stably transfected Schwann cells (data not shown). *In vivo*, we found that myelinating Schwann cells express this gene, which is of further interest considering recent data about the role of bHLH transcription factors and HLH proteins in oligodendrocyte differentiation (Kondo and Raff, 2000; Wang et al., 2001). Future experiments will reveal whether Mash2 is also expressed by oligodendrocytes and whether it affects their differentiation and/or proliferation. It will also be interesting to investigate whether the observed localization of a small fraction of Mash2 proteins outside of the nucleus is a sign of posttranslational regulation by means of protein shuttling, similar to what has been proposed for Krox24 (Topilko et al., 1997). Conversely, endothelial cells of nerve blood vessels were also found to express Mash2, but no apparent regulation after nerve lesion was observed. Because Krox24 is known to be expressed by endothelial cells and to be induced after vascular injury (Khachigian et al., 1996), it remains to be shown whether Mash2 also responds to such lesions.

As our results demonstrate, Mash2 can act as both a transcriptional repressor (for Krox24 and Mob-1) and activator (for CXCR4 and p57kip2), and it is conceivable that, depending on the dimerization partner(s) or the context of the regulatory element, Mash2-containing heterodimers exert different regulatory functions. However, it must be emphasized that there is no proof for direct interactions of the Mash2 protein with the four target genes. Nevertheless, we noticed that the genomic sequences of all four targets contain multiple E-box binding sites, some of them occurring in the 5′ upstream regions. Although their functionality has yet to be demonstrated, our data indicate that Mash2 controls their transcriptional activities. In this respect, it is important to note that the bHLH factors USF, c-myc, and YY1 were recently shown to regulate CXCR4 expression by interacting with an E-box located within the promoter region (Moriuchi et al., 1999).

The identification of downstream target genes is one way to reveal molecular pathways that Mash2 is acting on and provides evidence for possible cellular functions of this protein. Although our sorting procedure resulted in only an enriched but not in a pure population of transiently Mash2-transfected cells, we were able to demonstrate unambiguously that Mash2 is a specific regulator of the Krox24, Mob-1, CXCR4, and p57kip2 genes. The observed effect must be specific, because, for example, the

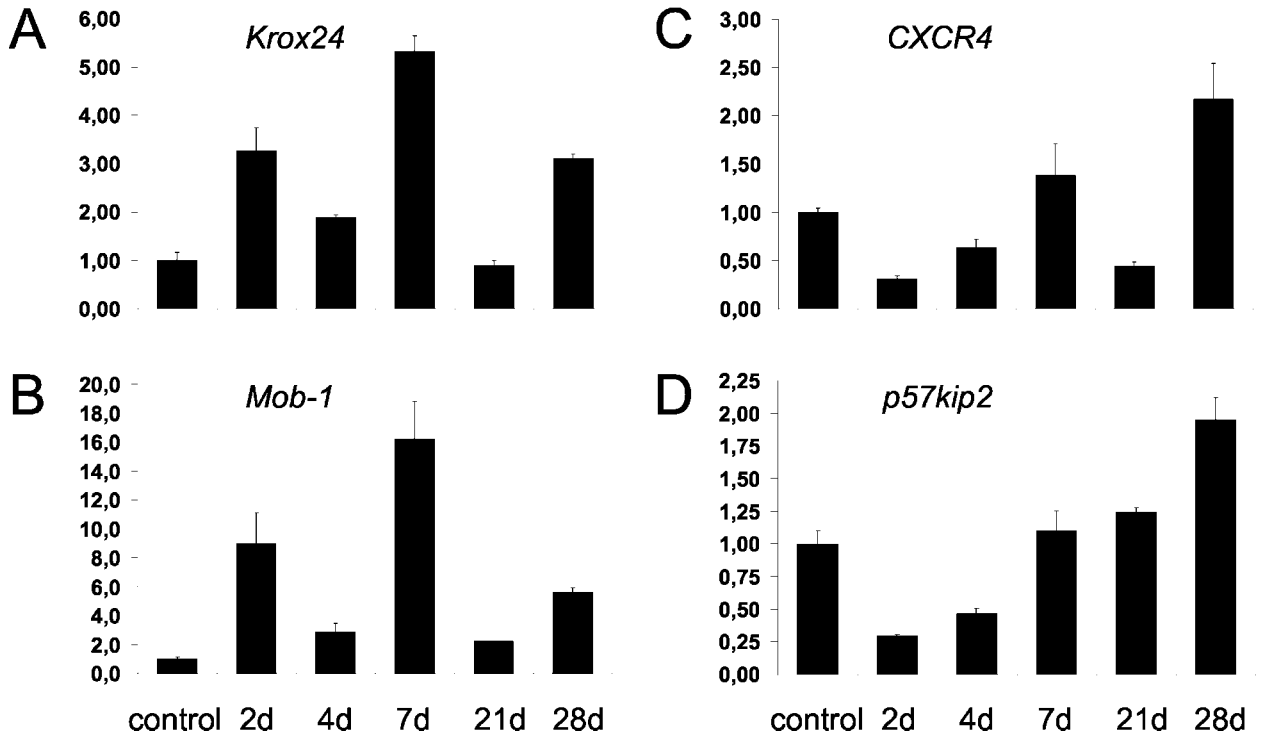

Figure 6. Quantitative RT-PCR was used to determine relative gene expression levels after sciatic nerve crush (control, unlesioned adult rat sciatic nerve; 2–28 d, distal nerve stumps of lesioned sciatic nerves 2–28 d after injury). *A–D,* In contrast to Mash2, both genes, Krox24 (*A*) and Mob-1 (*B*), displayed increased transcript levels after sciatic nerve crush, whereas CXCR4 (*C*) and p57kip2 (*D*) were transiently downregulated. One representative experiment of four is shown. GAPDH expression was used as reference, and data are mean values ± SD.

LNGFR gene was previously reported to be a direct target of bHLH proteins (Chiaramello et al., 1995) but was not affected by Mash2 in our assays (data not shown). Similarly, Krox24 was proposed to act on the expression of LNGFR gene. Nevertheless, when Mash2 suppressed Krox24 levels in our *in vitro* Schwann cell model, we found that LNGFR expression was not changed, which is in contrast to the antisense experiments performed by Nikam et al. (1995). During nerve development, Krox24 is found in Schwann cell precursors, and after a second phase of expression after birth, this gene becomes downregulated as myelination is initiated and resides in a small cellular subpopulation (Topilko et al., 1997). These authors suggested that these are nonmyelinating Schwann cells and Krox24 and Krox20 mutually suppressing each other. However, this latter assumption was shown by Nagarajan et al. (2001) to be wrong. They used a similar DNA array approach to identify Krox20 targets. Apart from myelin genes such as P0 and PMP22, which we found not to be affected by Mash2, NGFI-A (Krox24) was among the induced genes. Therefore, Mash2 is thus far the only known repressor of Krox24 in Schwann cells and can thus be regarded as being involved in the maturation process, but not necessarily as a myelination factor. Because we did not find LNGFR-positive Schwann cells that expressed Mash2, we conclude that this transcription factor is confined to the myelinating Schwann cell lineage. This indicates that Krox24 is probably not specific to non-myelin-forming cells and that it might be expressed at low levels in some myelinating cells.

Our experiments revealed that the CDK inhibitor p57kip2 is highly inducible by Mash2, and this is likely to be the reason for the reduced proliferation rate of Mash2-overexpressing cells. We have recently observed that after nerve crush, a number of cell cycle-specific proteins were transiently upregulated, among them cyclinD1, cyclinD4, and CDK4 (Bosse et al., 2001), providing additional evidence that tight control of Schwann cell proliferation is important for nerve regeneration. Interestingly, Mash2 and p57kip2 share a number of features, for example, their expression in spongiotrophoblasts (Takahashi et al., 2000) and colocalization in a cluster of imprinted genes on human chromosome 11p15.5 and on mouse distal chromosome 7 (Guillemot et al., 1995; Hatada and Mukai, 1995; Matsuoka et al., 1995). The analysis of p57kip2 knock-out mice and mutation screenings implied that this gene is involved in the pathology of the Beckwith–Wiedemann syndrome in humans, which is characterized by congenital malformations, organomegaly, and childhood neoplasms (Reik and Maher, 1997). Whether Mash2, as a potent activator of p57kip2 transcription, can be assigned a similar role is a tempting hypothesis and awaits future analysis.

Furthermore, we found that chemokine signaling in peripheral nerves is controlled by Mash2. The Mob-1 gene is the rat homolog of human and mouse IP10 and CRG2 genes, respectively, and a number of different studies have shown that this α-chemokine binds to the CXCR3 receptor, mediates the inflammatory response after tissue injury or infection, and acts as chemoattractant for activated T-lymphocytes and monocytes (Taub et al., 1993; Farber, 1997). IP10 was also proposed to play a role in smooth muscle cell migration and proliferation and in astrocyte chemoattraction (Wang et al., 1996, 1998). In the CNS, IP10 expression was found to be specifically induced both in focal stroke (Wang et al., 1998) and in multiple sclerosis (MS) lesions (Simpson et al., 2000). The finding that Schwann cells produce this proinflammatory cytokine is new and implies that Mob-1 secretion after peripheral nerve injury is involved in the onset of degenerative events that are crucial for nerve regeneration as

both macrophages and lymphocytes invade the injured nerve stump (Stoll et al., 1989; Moalem et al., 1999). Thus, Mash2 appears to be a Schwann cell-specific mediator of the injury-related immune response. Future experiments will reveal whether cells other than those of the immune system express the CXCR3 receptor and are thus likely to respond to Mob-1.

The CXCR4 receptor is the murine counterpart of fusin, an HIV-1 entry coreceptor that is naturally activated on binding of the α-chemokine SDF-1 (Bleul et al., 1996; Nagasawa et al., 1996). A number of different functions have been attributed to this signaling pathway, such as B-cell differentiation, T-lymphocyte attraction, and induction of neuronal apoptosis (Nagasawa et al., 1996; Aiuti et al., 1997; Hesselgesser et al., 1998). We recently demonstrated that the expression of the three splice variants SDF-1α, SDF-1β, and SDF-1γ is widespread and that gene transcripts are also found in oligodendrocytes and neurons of the adult brain and in Schwann cells of peripheral nerves, suggesting functions outside of the immune system (Gleichmann et al., 2000). The finding that CXCR4 is also expressed on Schwann cells implies autocrine or paracrine signaling mechanisms, and it is possible that the modulation of expression levels, such as the transient down-regulation after nerve lesion, is implicated in Schwann cell differentiation, proliferation, or survival.

In conclusion, we have shown that Schwann cell dedifferentiation and redifferentiation as it occurs in the injured peripheral nervous system is accompanied by significant changes in Mash2 expression. We show that Mash2 is a negative regulator of proliferation and provide strong evidence that in cultured Schwann cells and in the peripheral nerves, Mash2 is an upstream regulator of Krox24, Mob-1, CXCR4, and p57kip2. Future experiments will reveal whether Mash2 exerts a similar role in the myelinating glial cells of the CNS, and the construction and detailed analysis of conditional mouse mutants should elucidate the functional role or roles of Mash2 *in vivo*.

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