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A myelin protein is encoded by the homologue of a growth arrest-specific gene

(gene regulation/sciatic nerve/regeneration)

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ABSTRACT **Striking features of the cellular response to sciatic nerve injury are the proliferation of Schwann cells in the distal nerve stump and the downregulation of myelin-specific gene expression. Once the axons regrow, the Schwann cells differentiate again to reform the myelin sheaths. We have isolated a rat cDNA, SR13, which is strongly downregulated in the initial phase after sciatic nerve injury. This cDNA encodes a glycoprotein that shares striking amino acid similarity with a purified myelin protein and is specifically precipitated by a myelin-specific antiserum. Immunohistochemistry experiments using peptide-specific polyclonal antibodies localize the SR13 protein to the myelin sheath of the sciatic nerve. Computer-aided sequence analysis identified a pronounced homology of SR13 to a growth arrest-specific mRNA (Gas-3) that is expressed in resting but not in proliferating 3T3 mouse fibroblasts. SR13 is similarly downregulated during Schwann cell proliferation in the rat sciatic nerve. The association of the SR13 as well as the Gas-3 mRNA with nonproliferating cells in two different experimental systems suggests a common role for these molecules in maintaining the quiescent cell state.**

Intercellular communications, such as the interaction between neurons and glial cells, are an important aspect of the development of the nervous system. In the peripheral nervous system, the contact between Schwann cells and axons is crucial for the synthesis of the myelin sheath (1). This specialized membranous structure, consisting of proteins and lipids, increases the propagation velocity of action potentials along the axon (2). During development, Schwann cells cease proliferation concomitant to the initiation of myelin synthesis (3). This process is reversed in Wallerian degeneration when the Schwann cells downregulate myelin gene expression and resume proliferation (4, 5). If the injured peripheral nerve is allowed to regenerate, the Schwann cells will recapitulate the developmental program leading to functional recovery (6). Therefore, important insights into neuronal development can be gained by studying regeneration of the nervous system.

As outlined above, a key component of regeneration in the sciatic nerve is the proliferation of Schwann cells in the distal nerve stump of the injured nerve. The mechanisms that lead to the pronounced changes observed in Schwann cells after nerve injury are poorly understood. Several proteins are known to be upregulated or reexpressed by Schwann cells after sciatic lesions, including nerve growth factor (7), the nerve growth factor receptor (8), and some neuronal cell adhesion molecules like L1 and N-CAM (9, 10). In contrast, myelin genes are strongly repressed soon after injury (4, 11).

To get a better understanding of the molecular events underlying the mechanisms described above, we have constructed and screened rat sciatic nerve cDNA libraries to identify genes whose expressions are regulated during neuronal regeneration (12). This report describes the characterization¶ and expression of one of the isolated clones, termed SR13, which is highly regulated during sciatic nerve regeneration. We present evidence that the SR13 mRNA encodes a myelin protein and that the gene encoding this protein is highly related to a growth arrest-specific gene.

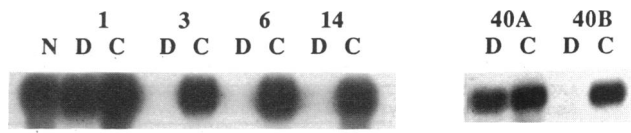

FIG. 1. Regulation of expression of the SR13 mRNA during sciatic nerve degeneration and regeneration. Total RNA (5 μg per lane) was analyzed by Northern blotting using the SR13 cDNA as a probe. Numbers above the lanes refer to days after sciatic nerve crush. RNA was isolated from normal sciatic nerve (N) or after sciatic nerve injury from the distal segment of the ipsilateral (D) or contralateral (C) rat sciatic nerve. Lanes 40A and 40B, RNA isolated 40 days after crush injury (40A) or cut injury (40B).

MATERIALS AND METHODS

Materials. Restriction enzymes and high/low RNA molecular size markers were purchased from Bethesda Research Laboratories (BRL). Chemicals were obtained from Sigma unless stated otherwise. ^{14}C-labeled protein molecular size markers were purchased from Amersham.

DNA Manipulations and Sequence Analysis. Both strands of the SR13 cDNA insert were sequenced by the dideoxynucleotide chain-termination method (13) using a Sequenase kit (United States Biochemical) and double-stranded plasmid DNA as templates. Sequencing primers were either the T7 promoter primer, the CDM8 reverse primer (InVitrogen, San Diego), or synthetic oligonucleotides from previously determined sequences. The sequences were analyzed by using the University of Wisconsin Genetics Computer Group programs (14). The homology to Gas-3 was identified by using the FASTA program to search the GenBank (Release 64.0), European Molecular Biology Laboratory (Release 23.0), and Swiss-Prot (Release 14.0) data bases; and the GAP program was used for alignment of sequences.

RNA Isolation and Northern Blotting. Total RNA was isolated by the method of Chomczynski and Sacchi (15) and examined by Northern blotting using Hybond membranes (Amersham). Electrophoresis, transfer of RNA, and hybridizations were done as described (12). ^{32}P-labeled SR13 cDNA

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M69139).

A

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   1 GGGGGAAGCCAGCAACCTAGAGGACGCCCCCGAGTTTGTGCCTGAGGCTACTCCGCTCTGAGC
  64 CGGCTGTCCCTTTGAACTGAAAGCACCGCTCCACCGAGCCCGAGCCCAACTCCAGCCACCATG
                                                                 Met
 127 CTTCTACTCTTGTTGGGGATCCTGTTCCTTCACATCGCGGTGCTAGTGTTGCTCTTCGTCTCC
     LeuLeuLeuLeuLeuGlyIleLeuPheLeuHisIleAlaValLeuValLeuLeuPheValSer
 190 ACCATCGTCAGCCAATGGCTCGTGGGCAATGGACACAGGACTGATCTCTGGCAGAACTGTACC
     ThrIleValSerGlnTrpLeuValGlyAsnGlyHisArgThrAspLeuTrpGlnAsnCysThr
 253 ACATCCGCCTTGGGAGCCGTCCAGCACTGCTACTCCTCATCTGTGAGCGAATGGCTTCAGTCT
     ThrSerAlaLeuGlyAlaValGlnHisCysTyrSerSerSerValSerGluTrpLeuGlnSer
 316 GTCCAGGCCACCATGATCCTGTCTGTCATCTTCAGCGTCCTGTCCCTGTTCCTGTTCTTCTGC
     ValGlnAlaThrMetIleLeuSerValIlePheSerValLeuSerLeuPheLeuPhePheCys
 379 CAGCTCTTCACTCTCACCAAAGGCGGCCGCTTTTACATCACTGGAGTCTTCCAAATCCTTGCT
     GlnLeuPheThrLeuThrLysGlyGlyArgPheTyrIleThrGlyValPheGlnIleLeuAla
 442 GGTCTGTGTGTGATGAGTGCAGCGGCCATCTACACAGTGAGACACAGTGAGTGGCATGTCAAC
     GlyLeuCysValMetSerAlaAlaAlaIleTyrThrValArgHisSerGluTrpHisValAsn
 505 AACGACTACTCCTATGGCTTTGCTTACATCCTGGCCTGGGTGGCTTTCCCGCTGGCCCTCCTT
     AsnAspTyrSerTyrGlyPheAlaTyrIleLeuAlaTrpValAlaPheProLeuAlaLeuLeu
 568 AGTGGCATCATCTACGTGATCCTGCGGAAACGCGAATGAGGCGCCCGACGCACCATCCGTCTA
     SerGlyIleIleTyrValIleLeuArgLysArgGluEnd
 631 GGCTCTGAGCGTGCATAGGGTACACAGGGAGGGAGGAAGGAAACCAGAAAACCAAACCAACCA
 694 ACCCAAAAGAGCTAGCCCCCAAACCCAAACGCAAGCCAAACCAAACAGAACACAGTTGAGTGG
 757 GGATTGCTGTCGATTGAAGATGTATATAATATCTATGGTTTATAAAACCTATTTATAACACTT
 820 TTTACATACATGTACATAGGATTGTTTGCTTTTTATGTTGACCGTCAGCCTCGTGTTGAATCT
 883 TAAACGACTCTACATCCTAACACTATAACCAAGCTCAGTATTTTCGTTTTGTTTCGTTTTTTT
 946 CATCTTTTTGTTTTGCTCAGACATAAAAAAAAAAAAAAAAATCCACGTGGCCCCCTTTCATCTG
1009 AAAGCAGATCCCTCCCTCCCATTCAACCTCATAGGATAACCAAAGTGCGGGGACAAACCCCAG
1072 ATGGCCAGAGGCCTTCACACTATGGGTGACCCAGTGAATTTAGCAGGAATAATCCGCTGCCCG
1135 AATCAATGTGTGAAGCCCTAAGCACTCACAGACGAAACGCCCTGACCAGAGCCCTCTGCGAAA
1198 CCAATAGCTGGTGGCTGCGGAACACTTGACCCTGAAGGCGGAGTACTGGGGCACATGTTTAAA
1261 TGAGACGTCAGAGACAAGCAATCTGTGAAATGGTGCTATAGATTTACCATTCCTTGTTATTAC
1324 TAATCATTTAAACCACTCACTGGAAACTCAATTAACAGTTTTATGACCTACAGCAGAACAGAG
1387 ACCCGATACAAACGGTTCGTAACTGCTTTCGTACATAGCTAGGCTGTTGTTATTACTACAATA
1450 AATAAATCTCAAAGCCTTCGTCACTCCCACAGTTTTCTCACGGTCGGAGCATCAGGACGAGGG
1513 TCTAGACCCTTGGGACTAGCAAATTCCCTGGCTTTCTGGGTCTAGAGTGTTCTGTGCCTCCAA
1576 GGACTGTCTAGCGATGACTTGTATTGGCCACCAACTGTAGATGTATATACGGTGTCCTTCTGA
1639 TGCTAAGACTCCAGACCTTTCTTGGTTTTGCTGGCTTTTTCTGATTTTATACCAACTGTGTGG
1702 ACTAAGATGCATTAAAATAAACATCAGAGTAACTC 1736
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B

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 1 MLLLLLGILFLHIAVLVLLFVSTIVSQWLVGNGHRTDLWQ 40
   |||||||||||||||||||||||||||||||||| |||||
 1 MLLLLLGILFLHIAVLVLLFVSTIVSQWLVGNGHTTDLWQ 40

41 NCTTSALGAVQHCYSSSVSEWLQSVQATMILSVIFSVLSL 80
   |||||||||||||||||||||||||||||||||||||| |
41 NCTTSALGAVQHCYSSSVSEWLQSVQATMILSVIFSVLAL 80

81 FLFFCQLFTLTKGGRFYITGVFQILAGLCVMSAAAIYTVR 120
   |||||||||||||||||||| ||||||||||||||||||||
81 FLFFCQLFTLTKGGRFYITGFFQILAGLCVMSAAAIYTVR 120

121 HSEWHVNNDYSYGFAYILAWVAFPLALLSGIIYVILRKRE 160
    ||||||| |||||||  ::  :||
121 HSEWHVNTDYSYGFATSWPGWPFP                 144
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C

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          1                                   17  40      44
SR-13     M L L L L L G I L F L H I A V L V / Q N C T T
          | | | | | | | | : : | ! | | | | |   | | | : |
PAS-II    M L L L L L G I I V L X V A V L V / Q N C S T
          | | | | | | | | : : | ! | | | | |   | | | : |
GAS-3     M L L L L L G I L F L H I A V L V / Q N C T T
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FIG. 2. (*A*) Sequence of the rat SR13 cDNA. Solid underlined regions correspond to putative membrane-spanning domains, whereas the broken line indicates the putative signal sequence. A N-linked glycosylation consensus site is marked with an arrowhead. (*B*) Amino acid sequence (one-letter code) comparison of rat SR13 (top sequence) and mouse Gas-3 (bottom sequence). Identical residues are indicated by solid vertical lines, and conservative amino acid substitutions are shown by colons. (*C*) Comparison of partial amino acid sequences of bovine myelin PAS-II, rat SR13, and mouse Gas-3. Numbering refers to amino acid positions in the SR13 protein. X represents an undetermined amino acid residue in the PAS-II protein sequence.

probes were prepared by using a hexanucleotide labeling kit (Boehringer Mannheim).

Cell Culture, Transfection, and Protein Analysis. The SR13 cDNA in the CDM8 expression vector (16) was transfected into COS-7 cells by a modification of the DEAE-dextran/chloroquine method (17). Transfected cells were metabolically labeled with [^{35}S]cysteine (Amersham) for 3 hr and lysed in phosphate-buffered saline containing 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, and 2 mM phenylmethylsulfonyl fluoride. Labeled proteins were immunoprecipitated by using either normal rabbit serum or a rabbit polyclonal antiserum raised against purified rat sciatic nerve myelin. Immunocomplexes were formed using Pansorbin cells (Calbiochem). Precipitated proteins were eluted and analyzed on an SDS/12.5% polyacrylamide gel under reducing conditions.

To inhibit N-linked glycosylation, transfected COS cells were metabolically labeled in the presence of tunicamycin (10 μg/ml), lysed, and analyzed as described above.

Peptide Antibodies and Immunohistochemistry. Two peptides corresponding to different hydrophilic regions of the cDNA-predicted SR13 protein sequence were synthesized on a Milligen/Biosearch automated peptide synthesizer, and rabbit antisera were raised (details will be presented elsewhere). Immunoperoxidase studies were performed on paraffin-embedded normal rat sciatic nerves fixed in 4% paraformaldehyde/0.1 M sodium phosphate, pH 7.4 by using the peroxidase-antiperoxidase method (18). Primary antisera were used at 1:150 dilutions.

RESULTS

Isolation and Regulation of SR13 mRNA. During the screening of a rat sciatic nerve cDNA library, an mRNA (SR13) was isolated that is abundantly expressed in sciatic nerve (representing 0.2% of the insert-containing library clones) and was strongly repressed following crush injury of the sciatic nerve. The longest SR13 cDNA insert [1.74 kilobases (kb)] was used to perform Northern blot analysis to determine its time course of expression during neuronal degeneration and regeneration (Fig. 1). The SR13 transcript (1.8 kb) was partially repressed within 1 day after sciatic nerve injury and became <5% of normal levels from days 3 through 14. Forty days after the initial lesion (when the regeneration process was complete), the expression level of SR13 returned to close to

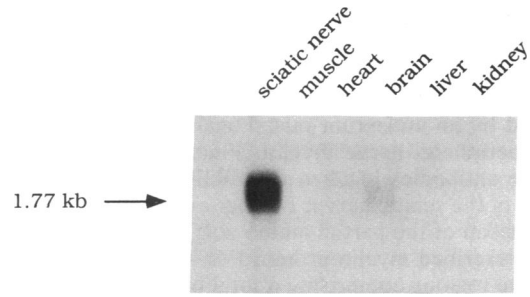

FIG. 3. Tissue distribution of rat SR13 mRNA. Each lane contains 10 μg of total RNA except for the sciatic nerve lane (2 μg of total RNA). mRNA sizes were determined by using RNA size markers.

normal (Fig. 1, lanes 40A). In contrast, if the nerve was cut and regeneration was prevented, SR13 was still downregulated at day 40 (Fig. 1, lanes 40B). The strong repression of this mRNA after sciatic nerve injury is strikingly similar to the patterns observed for the mRNAs encoding myelin P_0 and myelin basic protein (4, 11).

Analysis of SR13 Sequence. The nucleotide sequence of the 1.74-kb SR13 cDNA insert is shown in Fig. 2*A*. An open reading frame of 480 nucleotides was identified, which encodes a putative protein of 160 amino acid residues with a predicted molecular mass of ≈18 kDa. Based on hydropathy plots and secondary structure predictions, amino acid residues 1–26, 67–88, 98–118, and 134–155 represent four hydrophobic, possibly membrane-associated domains. The N-terminal sequence possesses the characteristics of a signal peptide with the predicted cleavage site at Ser-26/Gln-27 (according to the algorithm of von Heijne, ref. 19). There is a single consensus site for N-linked glycosylation at Asn-41. Comparison of the cDNA sequence to the GenBank data base (20) suggests that the SR13 mRNA is the rat homologue of the recently described growth arrest-specific mRNA (Gas-3), which was isolated from serum-starved 3T3 mouse fibroblasts (21, 22). Both sequences show an overall homology of 92% at the nucleotide level including the 3′ untranslated region. The two predicted proteins are 97% homologous through the first 135 amino acid residues but diverge at the C terminus (Fig. 2*B*). Interestingly, the additional C-terminal amino acids in SR13 are predicted to encode an extra, possibly membrane-spanning domain.

Expression of SR13 mRNA in Different Tissues. SR13 was strongly expressed in sciatic nerve as a 1.8-kb transcript, whereas a weak signal was detected in brain tissue (Fig. 3). Heart and muscle show traces of SR13-specific mRNA after prolonged exposure, probably reflecting the strong innervation of these organs.

Expression of Recombinant SR13 Protein in COS-7 Cells. The SR13 cDNA was inserted into the eukaryotic expression vector CDM8 and transiently expressed in COS-7 cells. Transfected cells were metabolically labeled, lysed, and immunoprecipitated with a polyclonal antiserum directed against purified peripheral nerve myelin. A 19-kDa protein was specifically detected in SR13-transfected COS cells (Fig. 4, lane A2). No labeled proteins were precipitated from either SR13-transfected COS cells incubated with normal rabbit serum or CDM8 (vector only)-transfected COS cells incubated with the specific antiserum (Fig. 4, lanes A1 and A3, respectively). The presence of an N-glycosylation on the 19-kDa protein was substantiated by treating SR13-transfected COS cells with the N-glycosylation inhibitor tunicamycin. Under these culture conditions, the molecular mass of the 19-kDa protein was reduced to 15 kDa (Fig. 4, lane B2).

These findings are consistent with the hypothesis that COS-7 cells both process the precursor to the mature protein (of molecular mass of ≈15 kDa) and add N-linked sugars (to increase the molecular mass of the N-glycosylated protein to ≈19 kDa). The results also demonstrate that the SR13 cDNA, identified solely with a reverse-genetic approach, encodes an expressed protein in normal sciatic nerve.

Localization of SR13-Like Immunoreactivity in Rat Sciatic Nerve. The immunoprecipitation of SR13-encoded protein by a myelin-specific antibody suggested that SR13 is a myelin protein. This hypothesis was confirmed by using polyclonal rabbit antisera raised against peptides derived from the cDNA-predicted SR13 amino acid sequence in an immunohistochemical analysis. Intense immunoreactivity was localized to the myelin sheath of the sciatic nerve (Fig. 5) in a pattern indistinguishable from the staining observed with antisera against myelin basic protein (data not shown). No immunoreactivity was detected in axons and connective tissue support cells. Skeletal muscle was also unstained except for innervating myelinated fibers, explaining the weak SR13-specific signals observed in Northern blots. Identical results were obtained with two antisera raised against two peptides from different regions of the SR13 protein.

DISCUSSION

This report describes the cloning and expression of the rat SR13 cDNA, which encodes an additional myelin protein. This cDNA is highly related to the growth arrest-specific gene Gas-3, isolated from resting mouse 3T3 fibroblasts.

The SR13 protein is a myelin protein based on the following evidence. First, the SR13 mRNA shows a similar time course

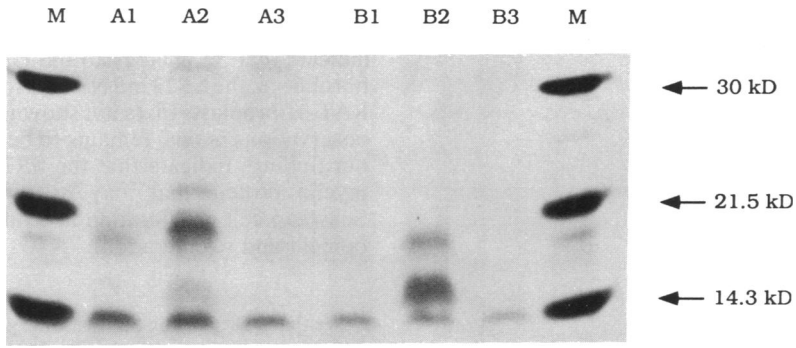

FIG. 4. Lanes A1–A3: SR13 protein expressed in COS cells is recognized by antiserum to rat myelin. Lane A1, COS were cells transfected with SR13 in the CDM8 expression vector, and proteins were immunoprecipitated with normal rabbit serum. Lane A2, proteins of the same transfected COS cells as in lane A1 were immunoprecipitated with the anti-sciatic nerve myelin serum. Lane A3, COS cells were transfected with the CDM8 vector only, and proteins were immunoprecipitated with the anti-sciatic nerve myelin serum. Lanes B1–B3: SR13 protein is N-glycosylated in COS cells. Transfected COS cells were grown in the presence of tunicamycin. Lane B1, COS cells were transfected with SR13 in the CDM8 expression vector, and proteins were immunoprecipitated with normal rabbit serum. Lane B2, COS cells were transfected with SR13 in the CDM8 expression vector, and proteins were immunoprecipitated with the anti-sciatic nerve myelin serum. Lane B3, COS cells were transfected with CDM8 only, and proteins were immunoprecipitated with the anti-sciatic nerve serum. M, protein molecular size markers.

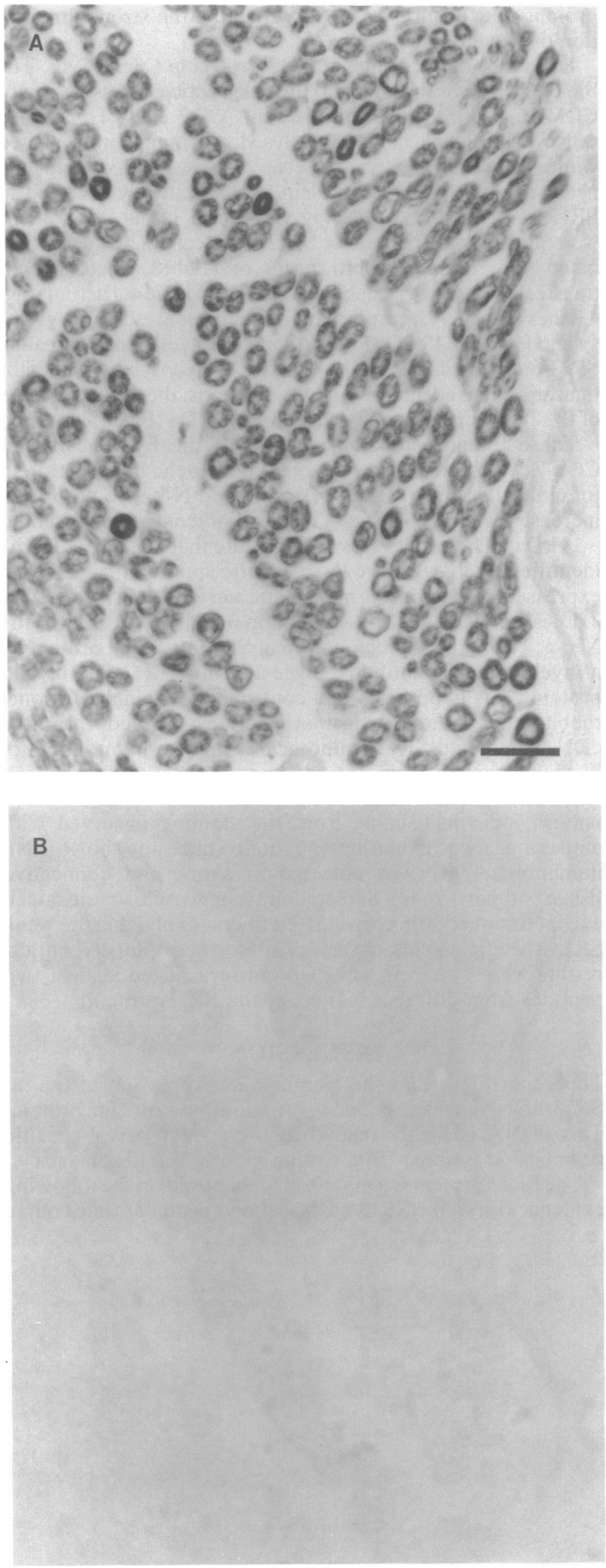

FIG. 5. Immunohistochemical localization of SR13-like immunoreactivity in cross sections of normal adult rat sciatic nerve stained with anti-SR13 peptide antiserum (*A*) or with peptide-preblocked anti-SR13 peptide antiserum (*B*). Each darkly stained toroidal structure in *A* represents the myelin sheath of a single axon. (Bar = 20 μm.) Controls using preimmune serum did not show any detectable staining (data not shown).

of downregulation after sciatic nerve injury as observed for the mRNAs encoding the classical myelin proteins P_0 and myelin basic protein. Second, the recombinant SR13-encoded protein expressed in COS cells is specifically recognized by an antiserum raised against preparations of purified peripheral nerve myelin. Finally, SR13-specific antipeptide antibodies localize the SR13 protein to the myelin sheath of the sciatic nerve. Further evidence emerges from a comparison of the partial amino acid sequence of the previously described myelin protein PAS-II (23) and SR13. Two separate regions comprising a total of 22 amino acids of the PAS-II protein have been sequenced (24). These sequences share >80% identity with the SR13 protein (Fig. 2*C*); the few exceptions may represent species differences between bovine PAS-II and rat SR13. PAS-II was isolated from purified myelin of various species (23) and shows the same developmental pattern of expression in the chicken sciatic nerve as other major myelin proteins (25). Based on these results, we conclude that PAS-II and SR13 are likely to be the same myelin protein. In addition, these proteins may be identical to a 19-kDa rat myelin protein (26, 27).

The N-terminal amino acid sequences derived from PAS-II as well as SR13 suggest that the putative signal sequence, in contrast to COS cells, appears not to be cleaved in Schwann cells (Fig. 2*C*). Similarly, Manfioletti *et al.* (22) failed to see signal-peptide cleavage when the Gas-3 mRNA was translated in a cell-free system. Whether these apparent discrepancies are due to differences between the experimental systems, interspecies variations, or both remains to be determined.

Importantly, the rat SR13 mRNA shows a high degree of sequence identity to the mouse Gas-3 mRNA, suggesting that these molecules are homologues. The SR13/Gas-3 mRNAs belong to a group of genes whose expression is specifically associated with the quiescent cell state. Such transcripts have been isolated from a variety of cell lines including 3T3 mouse fibroblasts (Gas genes; ref. 21), chinese hamster ovary cells (GADD genes; ref. 28) and TA1 adipocytes (29). The function of these mRNAs is not known, but an attractive hypothesis is that these molecules and/or their encoded putative proteins are directly involved in the regulation of general cell growth. To our knowledge, this is the first report that describes the isolation of one of these growth arrest-specific RNAs from normal tissue and its expression at the protein level *in vivo*. We have demonstrated that the SR13 mRNA is translated to a myelin membrane glycoprotein in differentiated, quiescent Schwann cells. After nerve injury, the SR13 mRNA is rapidly downregulated at the time of Schwann cell proliferation. Similarly the homologous Gas-3 mRNA is abundantly expressed in differentiated nonproliferating fibroblasts, yet is downregulated when the fibroblasts are induced to proliferate by addition of serum (22). Preliminary results indicate that, in analogy to the Gas-3 mRNA in mouse 3T3 fibroblasts, the SR13 mRNA is also induced in serum-starved RAT-2 fibroblasts (data not shown). The significance of these observations *in vivo* remains to be determined. In summary, our findings indicate that the SR13 protein is an additional myelin protein that may be involved in the control of Schwann cell proliferation and differentiation during development and regeneration.

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