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Axon-regulated expression of a Schwann cell transcript that is homologous to a 'growth arrest-specific' gene

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We have isolated a 1.8 kb cDNA (pCD25) clone that encodes a transcript that is differentially expressed during nerve regeneration. Nucleotide sequence comparison indicates 89.6% homology with the recently identified murine 'growth arrest-specific' gene *gas3*. The open reading frame of the *CD25* transcript predicts a 17 kDa protein with four putative transmembrane regions. Steady-state levels of the *CD25* mRNA are very much higher in sciatic nerve than in other tissues, and expression in sciatic nerve is confined to Schwann cells. Following nerve injury, the transcript levels rapidly declined in nerve segments distal to the site of lesion, but recovered upon nerve regeneration. In contrast, in distal stumps of permanently transected nerves, the mRNA level remained very low. Substantial amounts of the mRNA could be reinduced only upon anastomosis of these interrupted nerve stumps. Re-induction of the mRNA followed the elongation of regenerating axons through the distal nerve segment. Our data indicate that axons regulate expression of the *CD25* mRNA in Schwann cells, and suggest that the CD25 protein functions during Schwann cell growth and differentiation.

Key words: growth arrest-specific gene/nerve injury/nerve regeneration/Schwann cell/sciatic nerve

Introduction

Interruption of axons in the peripheral nervous system of mammals leads to a complex but stereotyped sequence of histopathological reactions (Wallerian degeneration) including disintegration of the distal axon segment, fragmentation of myelin sheaths and mitotic division of Schwann cells (for review, see Sunderland, 1978). Presumably most of these events are the result of intricate interactions among different cell populations including neurons, Schwann cells, endoneurial fibroblasts, and endothelial cells normally present in peripheral nerves, as well as the hematogenous cells (e.g. macrophages) that infiltrate damaged nerves.

The molecular mechanisms underlying these cellular interactions are still poorly understood. Recently, several laboratories have investigated the altered expression of sheath cell proteins following nerve injury, for example myelin proteins (Lemke, 1988), growth factors and their receptors (Heumann *et al.*, 1987; Lindholm *et al.*, 1987; Meier *et al.*, 1989) as well as apolipoproteins (Ignatius *et al.*, 1986; Müller *et al.*, 1986; Spreyer *et al.*, 1990). The expression of these gene products by resident or non-resident cells in the nerve sheath, respectively, is thought to contribute to the supportive environment for axonal regeneration and nerve maturation. In this respect, the interrelationship between axons and their ensheathing Schwann cells is of particular interest.

It has been demonstrated that axons strongly influence Schwann cell reaction as diverse as stimulation of cell proliferation (Spencer *et al.*, 1981), the expression of basal lamina constituents (Bunge *et al.*, 1986), and the regulation of major myelin and NGF receptor genes (Lemke and Chao, 1988).

In the present paper we report on: (i) the cloning of a cDNA (pCD25) from crushed rat sciatic nerve that shows substantial sequence homology to a recently reported 'growth arrest-specific' gene of mouse; (ii) the tissue specificity of *CD25* transcript expression; (iii) the distinct cellular localization of *CD25* mRNA in rat sciatic nerve; (iv) the spatio-temporal pattern of changes in steady-state transcript levels in regenerating and non-regenerating injured nerve segments using three different experimental paradigms (crush, transection, anastomosis); (v) the expression of *CD25* mRNA during postnatal development of rat sciatic nerve; and (vi) the effect of forskolin on the steady-state concentration of this transcript in cultured cells.

Results

Differential screening of an Okayama–Berg cDNA library constructed with poly(A)$^+$ RNA derived from crushed rat sciatic nerve yielded 12 individual clones or groups of cross-hybridizing clones, respectively, which appeared to be regulated during peripheral nerve regeneration (Spreyer *et al.*, 1990). Two of these cDNA clones (pCD25, pCD3) represent a transcript which was markedly down-regulated one week after crush injury.

The nucleotide sequence of the pCD25 clone, as determined by sequencing both strands, and the deduced amino acid sequence are shown in Figure 1A. Computer-assisted sequence comparison indicates significant homology (89.6% on the nucleotide level, 81% on the amino acid level) to the 'growth arrest-specific' gene *gas3* that was recently identified in mouse fibroblasts (Manfioletti *et al.*, 1990). The open reading frame of pCD25 encodes a 17 kDa protein that contains four hydrophobic amino acid stretches (underlined in Figure 1A) as revealed by hydropathy index computation using SOAP (Klein *et al.*, 1985) predicting four putative transmembrane domains. A potential signal peptide cleavage

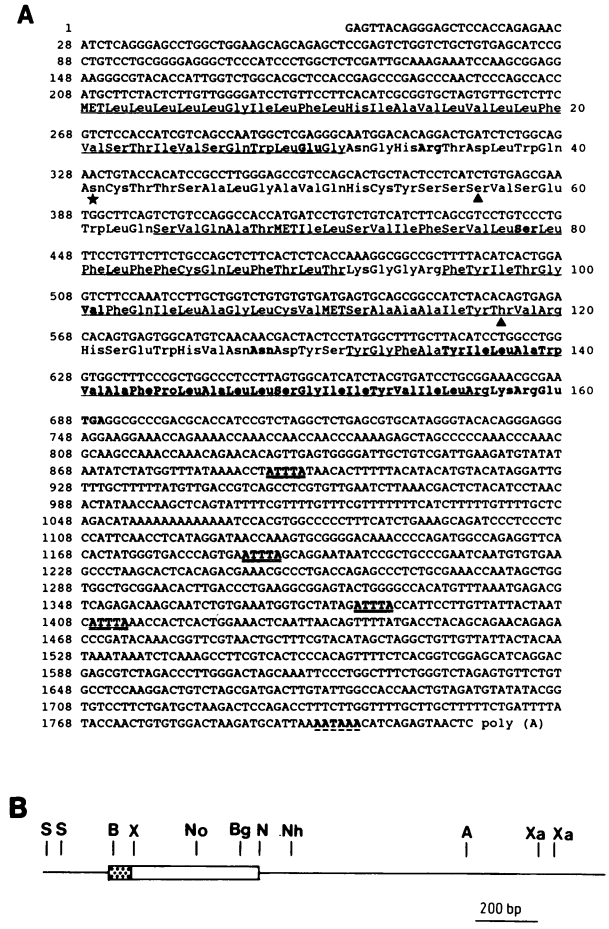

Fig. 1. Nucleotide sequence of pCD25 cDNA and deduced amino acid sequence. (**A**) A continuous open reading frame is encoded by nucleotides 208–687. The first start codon in the proximity of the 5′ end appears in the favourable nucleotide context (GCCACC-ATG-C) of the Kozak (1989) consensus sequence for translation initiation. Four hydrophobic amino acid stretches predicting putative transmembrane domains are underlined. The N-terminal hydrophobic region contains a potential leucine-rich signal peptide sequence comprising amino acids 1–26. Differences in the amino acid sequence compared to the deduced sequence of *gas3* (Manfioletti *et al.*, 1990) are indicated in bold letters. A putative glycosylation site (Asn41) or phosphorylation sites (Ser57, Thr118) are indicated by a star and triangles, respectively. Double lines indicate the AUUUA (ATTTA) pentamer motifs common in labile mRNAs and the dashed line marks the polyadenylation signal (AATAAA). (**B**) Schematic representation and restriction map of the pCD25 cDNA. The cDNA clone contains a 5′ leader sequence of 207 nucleotides followed by 480 nucleotides coding for the protein (box). The stippled section of the box represents a 78 nucleotide fragment encoding a potential signal peptide. The 3′ non-coding region extends for 1129 nucleotides. Abbreviations of restriction sites are A (*Aat*II), B (*Bam*HI), Bg (*Bgl*I), N (*Nar*I), Nh (*Nhe*I), No (*Not*I), S (*Sac*I), X (*Xho*I) and Xa (*Xba*I).

site (von Heijne, 1984) was identified between Ser26 and Gln27. The position of the start codon appears in the favourable context of the Kozak consensus for translation initiation (Kozak, 1989). Computer analysis further revealed a putative glycosylation site at Asn41 and two potential phosphorylation sites at positions Ser57 and Thr118.

In comparison to the deduced mouse gas3 amino acid sequence (Manfioletti *et al.*, 1990) we found only five single amino acid exchanges (Glu30, Arg35, Ser79, Val101 and Asp128) up to Ala135. Beyond that point an additional T in nucleotide position 613 of the pCD25 cDNA produces a frameshift that interrupts the amino acid homology for the

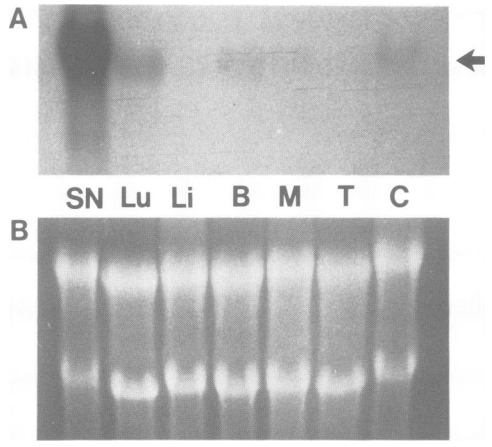

Fig. 2. (**A**) Northern blot analysis showing the abundance of the pCD25 transcript in different tissues. 8 μg of total RNA derived from sciatic nerve (SN), lung (Lu), liver (Li), brain (B), skeletal muscle (M), testis (T) and colon (C) of adult rats were hybridized with a [^{32}P]dCTP-labelled 1.1 kb fragment of pCD25 cDNA. The arrow indicates the position of the 18S (1.87 kb; Chan *et al.*, 1984) ribosomal RNA. (**B**) Ethidium bromide staining of RNA samples on the gel prior to Northern blot transfer to nitrocellulose.

entire C terminus. This frameshift has also been detected in the sequence of both strands of the independent pCD3 clone (not shown). In fact, this frameshift extends the C terminus of the Schwann cell protein for 16 amino acids beyond that of gas3 introducing an additional putative transmembrane domain.

Northern hybridization of a [^{32}P]dCTP-labelled 1.1 kb *Not*I–*Exo*III fragment (extending from nucleotide 486 to 1586) of the pCD25 cDNA to total RNA isolated from mature intact rat sciatic nerve detected a transcript of ~1.9 kb (Figure 2). Taking into account that poly(A) tails usually extend for ~100 residues, the cDNA clone shown in Figure 1 is likely to be full length.

Compared with the very strong Northern signal obtained using RNA from sciatic nerve only weak signals were detected with total RNA derived from lung and colon, whereas even lower signals were obtained with RNA from liver, brain, skeletal muscle and testis of adult rat (Figure 2). Densitometric evaluation of hybridization signals revealed that the abundance of *CD25* mRNA in sciatic nerve is at least one order of magnitude higher than in lung tissue, whereas this transcript is 50- and 100-fold less abundant in colon and brain, respectively, than in peripheral nerve.

We have further analysed temporal changes in the relative abundance of the transcript in sciatic nerve by Northern blots following two types of lesions: (i) crush injury, which leads to Wallerian degeneration in the distal nerve segment prior to regeneration of axons from the proximal stump into the distal segment; and (ii) transection of nerve and permanent separation of both stumps in order to prevent axonal growth into the degenerating distal stump.

In the proximal segments of both crush-lesioned and transected sciatic nerves only minor changes in mRNA abundance could be observed for at least 6 and 4 weeks after injury, respectively (Figure 3A,C). However, in the distal segments of crushed and transected nerves the transcript levels rapidly declined to very low values within 2 days or one week after lesion, respectively (Figure 3B,D). During the second week after a crush injury the transcript levels

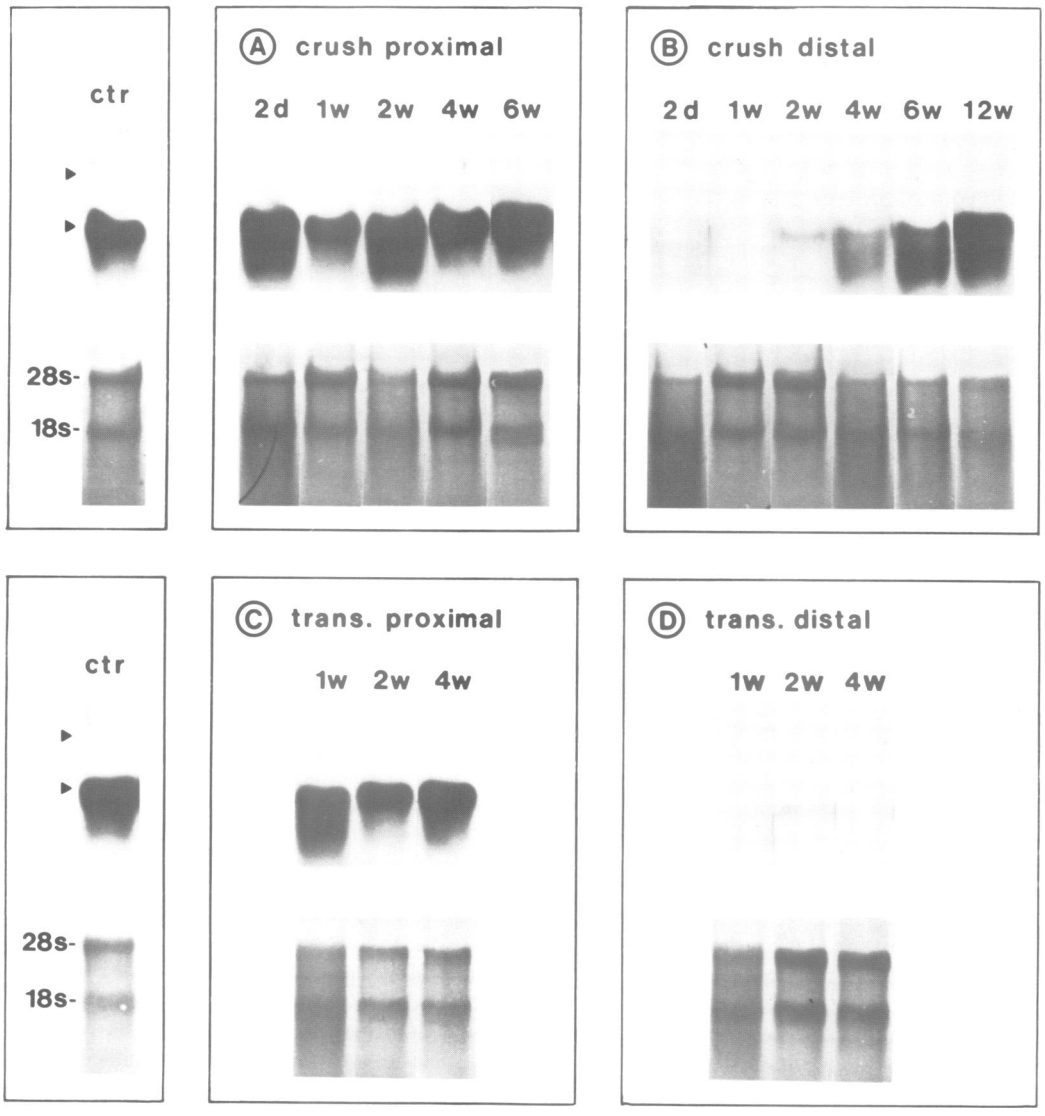

Fig. 3. Relative changes in steady-state levels of the Schwann cell transcript in proximal (**A**,**C**) and distal (**B**,**D**) segments of injured rat sciatic nerve. Nerves were either crushed (**A**,**B**) or transected (**C**,**D**) and total RNA was prepared from each time point indicated and 10 μg were hybridized to the [^{32}P]dCTP-labelled pCD25 cDNA probe (upper traces). For details see Materials and methods. Arrows indicate the positions of the 28S and 18S ribosomal RNAs. Lower traces show the methylene blue staining of the Nytran filter prior to hybridization. Ctr, non-injured control nerve.

began to rise again, reaching near control values at ~6 to 12 weeks post lesion. In contrast, in the distal stump of transected sciatic nerve, which was prevented from regeneration, the steady-state mRNA level stayed low for at least 4 weeks after axotomy (Figure 3D).

In situ hybridization of a digoxygenin–UTP-labelled antisense RNA probe to transverse sections derived from non-injured mature rat sciatic nerve revealed that specific hybridization signals were confined to S100-immunoreactive (IR) Schwann cells (Figure 4A,B). No hybridization signals were detectable in control experiments using a digoxygenin–UTP-labelled sense RNA probe (Figure 4C).

In transected nerves the transcript was still detectable by *in situ* hybridization in Schwann cells of the proximal stump which contains neurofilament-IR axons (Figure 5A,C). On the other hand, no hybridization signals could be identified in the permanently degenerated, axonally deprived, distal stump at 6 weeks after injury (Figure 5B,D).

In order to test the hypothesis that re-induction of the transcript in the distal nerve segment of crushed nerve (see Figure 3B) could be related to the regeneration of axons into this nerve portion, we have reconnected permanently degenerated distal stumps of transected nerves with their proximal (nondegenerated) counterparts 6 weeks after the initial lesion. Cross sections obtained at different time periods (1, 2 and 4 weeks) after reconnection as well as from different levels (5 and 25 mm from the site of anastomosis) along the distal nerve stump were processed for *in situ* hybridization and neurofilament immunohistochemistry. One week after reconnection neurofilament-IR axons, but no hybridization signals, were detectable 5 mm distal to the site of anastomosis (Figure 5E–H). Two weeks after anastomosis neurofilament-IR axons became visible 25 mm down the distal nerve stump, whereas hybridization signals were detectable at a distance of 5 mm from the site of anastomosis (Figure 5I–L). Four weeks after reconnection the hybridization signals appeared further down the regenerating distal nerve stump (Figure 5M–P). Hence,

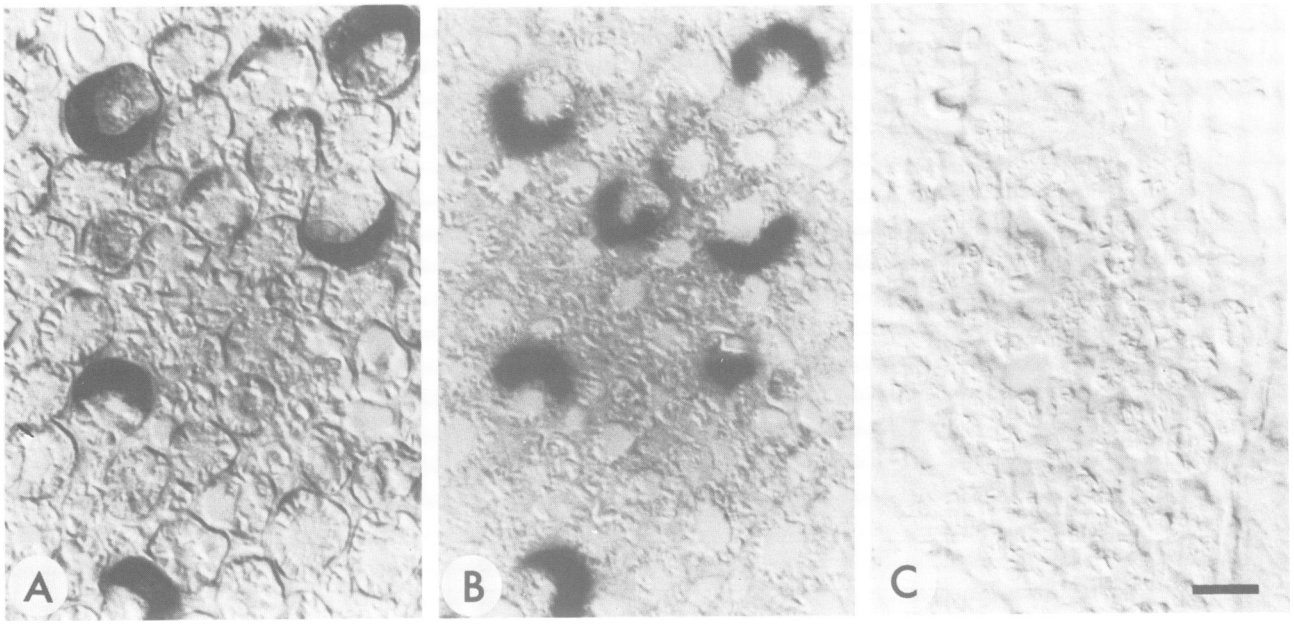

Fig. 4. Cellular localization of the transcript in paraffin sections of non-injured sciatic nerve of adult rat by *in situ* hybridization.
(**A**) Immunohistochemical identification of Schwann cells using antibodies to S100 protein. (**B**) *In situ* hybridization with a digoxygenin–UTP-labelled antisense RNA of an adjacent section to (A). Note that the hybridization signals are confined to cell bodies of S100-immunoreactive Schwann cells. (**C**) Hybridization of a digoxygenin–UTP-labelled sense RNA probe as negative control to a section from the same nerve. Bar in (C) = 10 μm.

growing axons entering the distal nerve stump clearly preceded the re-induction of the transcript in sheath cells along the same nerve segment.

During postnatal development of sciatic nerve, elevated steady-state levels of the *CD25* mRNA were already apparent at postnatal day 1, as demonstrated by Northern blot analysis (Figure 6). The elevated transcript level was maintained throughout maturation of the nerve.

When Northern blots were performed with RNA derived from confluent monolayers of cultured cells and cell lines, similar levels of the transcript could be detected in Schwann cells derived from neonatal rat sciatic nerve, the C6 CNS glioma cell line, the putative neural tumor cell line B103 and the Rat-2 fibroblast cell line, whereas the mRNA level in cerebral astrocytes appeared to be significantly lower (Figure 7). When forskolin (20 μM), a specific activator of adenylate cyclase, was added to the confluent cell cultures for 36 h, the *CD25* mRNA level was markedly up-regulated in Schwann cells but down-regulated in B103 cells and Rat-2 fibroblasts, whereas no changes in transcript levels were detectable in astrocytes and C6 glioma cells (Figure 7).

Discussion

We have cloned a cDNA (pCD25) complementary to a 1.9 kb transcript that is differentially expressed in Schwann cells during peripheral nerve regeneration in rat. Significant (89.6%) nucleotide sequence homology with the recently identified murine 'growth arrest-specific' gene *gas3* (Manfioletti *et al.*, 1990) suggests that rat *CD25* and mouse *gas3* are probably species variants of the same gene. The deduced amino acid sequence of *CD25* predicts a 17 kDa protein with four putative transmembrane regions. The positions of the first three hydrophobic regions of *CD25* are identical to those found in the *gas3* sequence. However, the fourth hydrophobic domain that appears at the C terminus of the Schwann cell protein due to a frameshift in the nucleotide sequence is not present in *gas3*.

Previous *in vitro* translation experiments with mouse *gas3* mRNA in the presence of microsomal preparations (Manfioletti *et al.*, 1990) suggested that this is an integral transmembrane protein. Hence, it is very likely that the homologous CD25 protein of rat peripheral nerve is expressed in the plasma or myelin membrane of Schwann cells. Confirmation of this hypothesis awaits the preparation of specific antibodies for immunohistochemistry.

The *CD25* transcript is most abundant in intact sciatic nerve but is very low in other tissues including the central nervous system, suggesting a high degree of tissue specificity of expression in adult rat. It is interesting to note that lung and colon have previously been considered as tissues with comparatively high levels of *gas3* mRNA in mouse (Manfioletti *et al.*, 1990).

Axotomy led to a rapid and very marked decline in transcript levels in distal nerve segments undergoing Wallerian degeneration, whereas in non-degenerating proximal nerve stumps the mRNA levels did not change significantly. By means of three different experimental paradigms we provide evidence that re-expression of the transcript in injured peripheral nerve is closely related to axonal regeneration: (i) in the distal segment of crushed nerve, which is known to regenerate, the transcript levels steadily increased reaching control values of normal nerve within ~6 weeks post-lesion; (ii) conversely, in the distal segment of transected nerve, where no axonal regeneration occurred, the mRNA steady-state concentration did not return to normal levels; and finally (iii) when a permanently denervated distal stump was reconnected with its non-degenerated proximal nerve segment by anastomosis, the transcript became re-expressed in those portions of the distal stump which had received regenerating (neurofilament-IR) axons.

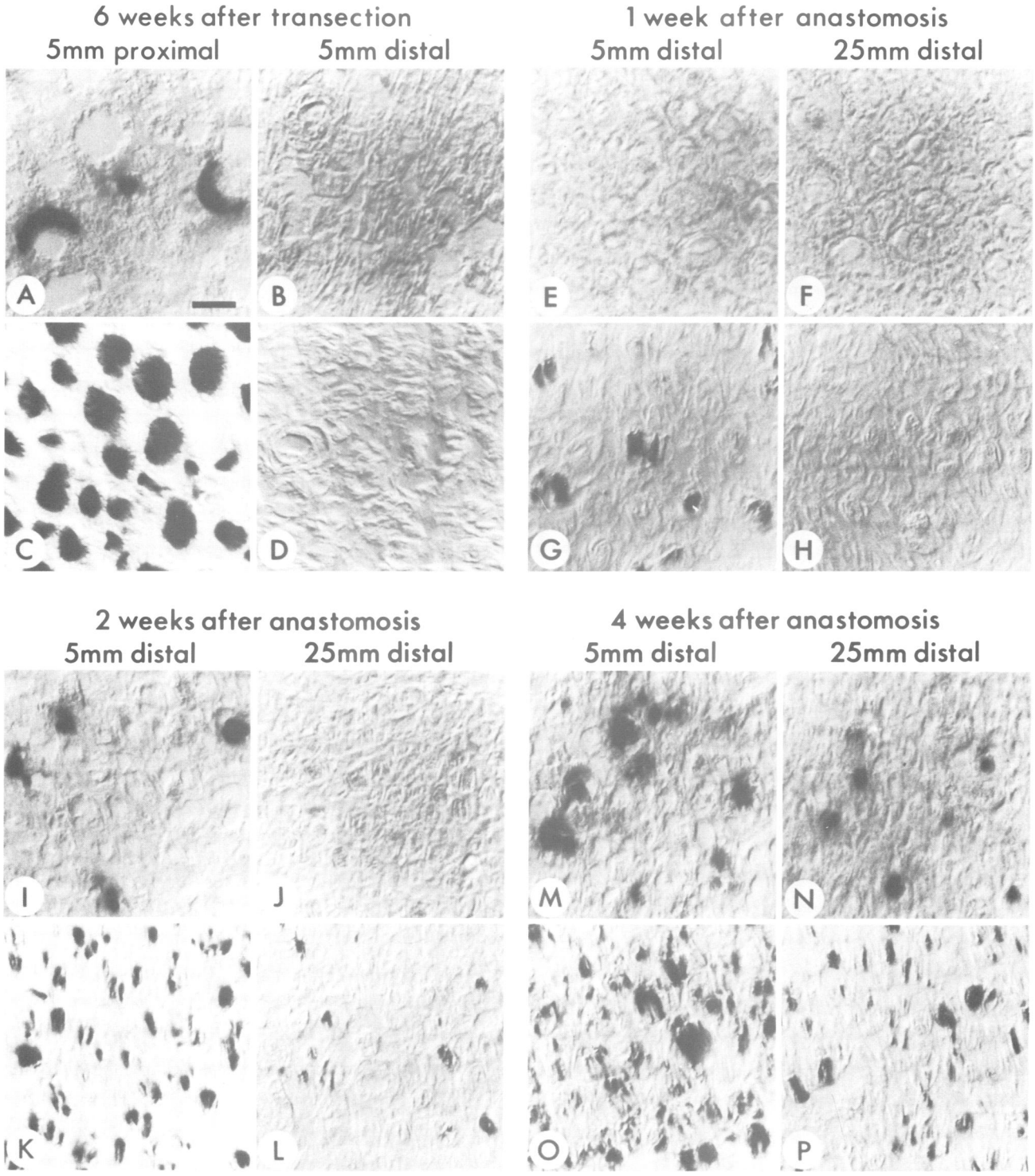

Fig. 5. Regional and temporal distribution of the transcript in comparison to the regeneration of neurofilament-immunoreactive (IR) axons following sciatic nerve transection and delayed anastomosis of the separated nerve stumps. (**A–D**) *In situ* hybridization using a digoxygenin–UTP-labelled antisense RNA probe (A,B) and neurofilament-IR (C,D) in paraffin sections obtained from the proximal (A,C) and distal (B,D) stumps at 6 weeks after transection. Distance of each tissue section from the site of injury in (A–D) is 5 mm, respectively. (**E–H**) *In situ* hybridization (E,F) and neurofilament-IR (G,H) in sections obtained from the distal nerve stump at one week after anastomosis. (**I–L**) *In situ* hybridization (I,J) and neurofilament-IR (K,L) in sections obtained from the distal stump at 2 weeks after anastomosis. (**M–P**) *In situ* hybridization (M,N) and neurofilament-IR (O,P) in sections obtained from the distal stump at 4 weeks after anastomosis. Distance of paraffin sections in (E–P) from the site of anastomosis, 5 mm and 25 mm, respectively. Note that the proximo-distal appearance of neurofilament-IR axons in the distal nerve stump clearly precedes the detection of the transcript in sheath cells along the nerve. Bar in (A) = 10 μm.

Our results suggest that Schwann cells in association with axons express high levels of the CD25 transcript, whereas interruption of this relationship leads to down-regulation of the mRNA level. Restoration of Schwann cell–axon contact in regenerating nerve may, in turn, re-establish the elevated transcript levels of noninjured nerve. This view is further

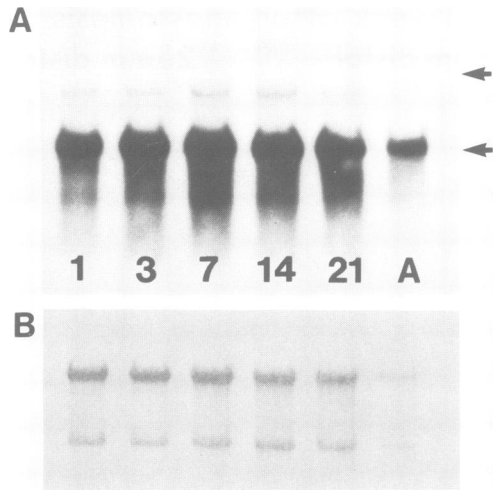

Fig. 6. Northern blot analysis of developing rat sciatic nerve. (**A**) 8 μg of total RNA derived from sciatic nerves of adult (A) and newborn Wistar rats at days 1, 3, 7, 14 and 21 postnatal were hybridized to [^{32}P]dCTP-labelled cDNA. Arrows indicate the positions of the 28S and 18S ribosomal RNAs, respectively. (**B**) Methylene blue staining of the filter prior to hybridization. Note that in lane (A) there is much less RNA and that in lane 7 there is slightly more RNA than in the other lanes.

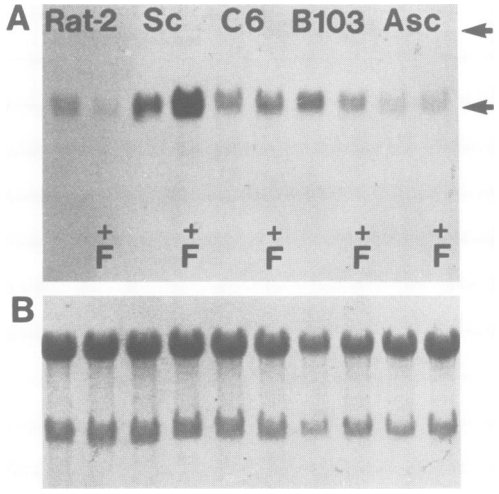

Fig. 7. Expression of the transcript in cultured cells. (**A**) Northern blot analysis of total RNA (6−8 μg) derived from Schwann cells (Sc), astrocytes (Asc), and Rat-2 fibroblasts, C6 glioma and B103 neural tumour cell lines hybridized to [^{32}P]dCTP-labelled cDNA. One of each pair of sibling cultures was pretreated with forskolin (+F, 20 μM) for 36 h. The positions of 18S and 28S ribosomal RNA are indicated by arrows. (**B**) Methylene blue staining of the Nytran filter prior to hybridization.

supported by the fact that in proximal stumps of injured sciatic nerve, where Schwann cells remain in close contact with their axons, the transcript levels stayed at normal high levels for at least 6 weeks after injury.

Regulation of Schwann cell gene expression by axons has been described previously, e.g. for myelin protein genes (Lemke and Chao, 1988; Trapp *et al.*, 1988) and the nerve growth factor (NGF) receptor (DiStefano and Johnson, 1987). Whereas expression of mouse myelin basic protein (MBP), rat protein zero (P_0) or human NGF receptor mRNA is regulated on the transcriptional level (Monuki *et al.*, 1990), experiments by Ciccarelli *et al.* (1990) suggest that the expression of *gas3* in murine fibroblasts is regulated on the post-transcriptional level. Evidence for post-transcriptional regulation is further provided by the fact that both *gas3* and the homologous *CD25* mRNA contain several AUUUA pentamers in their 3′ untranslated region that are common structural elements of labile mRNAs that are targets for specific cytosolic proteins causing rapid mRNA decay (Malter, 1989).

Cyclic AMP has been considered previously as a potential mediator of axonal influences on Schwann cell gene expression (Lemke and Chao, 1988). The present data suggest that cAMP is a likely second messenger candidate elevating the *CD25* transcript levels in Schwann cells (see Figure 7). In this respect it is important to note that regulation of the transcript seems to be different in cultured Schwann cells and in fibroblasts following forskolin treatment (see Figure 7).

Manfioletti *et al.* (1990) have demonstrated that the expression of *gas3* mRNA in 3T3 fibroblasts correlates with growth arrest initiated by either serum starvation or contact inhibition in confluent cell cultures. Schwann cell multiplication in peripheral nerve, which in rodents is intense neonatally, virtually ceases in adult animals (Asbury, 1967; Terry *et al.*, 1974). However, these cells retain their capacity to proliferate for up to 5−12 days in response to axonal interruption, whereas in permanently denervated distal stumps of transected nerve Schwann cell division has ceased (Spencer *et al.*, 1981). The elevated *CD25* transcript level in mature intact sciatic nerve (see Figures 2 and 3) that lacks significant Schwann cell multiplication, and the rapid decline in mRNA abundance following nerve injury (see Figure 3B,D) which initiates Schwann cell division, would be consistent with the hypothesis that expression of the *CD25* mRNA in Schwann cells may be 'growth arrest-specific'. However, high-level expression of the *CD25* mRNA in sciatic nerve of neonatal animals (see Figure 6), where Schwann cell proliferation is very intense, as well as the persistent low-level expression of this mRNA in permanently denervated distal stumps of transected nerve (see Figures 3D and 5), where Schwann cell division has ceased, is *not* consistent with a 'growth arrest-specific' expression of the transcript in the nervous system.

The biological function of this protein in the peripheral nervous system is not known but could be related to Schwann cell growth and differentiation. We speculate that *CD25* may represent a new myelin-associated protein for the following reasons: (i) the transcript is expressed at high steady-state levels in Schwann cells of mature peripheral nerve; (ii) the amino acid sequence predicts a membrane protein; (iii) the time course of changes in the steady-state mRNA level following nerve transection closely resembles the changes previously observed for transcripts of established myelin proteins (Trapp *et al.*, 1988); (iv) axons stimulate the expression of *CD25* mRNA as was shown for the myelin genes P_0 and MBP (Lemke and Chao, 1988); and (v) agents that increase the intracellular concentration of cAMP up-regulate mRNA levels of *CD25* as well as those of myelin proteins (Lemke and Chao, 1988). On the other hand, the high level expression of the transcript in neonatal rat sciatic nerve prior to the onset of myelination and the expression of *CD25* mRNA in a number of non-neuronal tissues, cultured astrocytes and fibroblasts clearly shows that the

CD25 gene product does not behave as a typical myelin protein and that it is not unique to myelinating cells.

Materials and methods

Animals and surgery

Adult Wistar rats (180–240 g) were anaesthetized with chloralhydrate (350 mg/kg body weight) administered intraperitoneally. Sciatic nerves were either crushed with jeweller's forceps or transected with a fine pair of scissors at upper thigh level. In order to prevent regeneration, transected nerve stumps were tied with surgical silk. Prior to RNA preparation from nerve stumps the lesion zone (2–3 mm from each segment adjacent to the site of injury) was removed and discarded.

In some experiments sciatic nerves were transected at mid-thigh level and the proximal and distal stumps were tied and kept separate for 6 weeks. Then the ends of the proximal and distal stumps of a transected nerve were cut off and the nerve endings were aligned and connected (anastomosis) using three to five sutures of 9-0 polyglactin fibres (Ethicon).

Cell culture

Cell cultures were prepared as described previously (Monuki *et al.*, 1989). Briefly, cell lines (Rat-2 fibroblasts, C6 glioma, B103 neural tumour line) and primary cerebral astrocytes were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin. Schwann cell cultures were prepared from neonatal rat sciatic nerve, purified and expanded using glial growth factor and 2 μM forskolin as described previously (Lemke and Chao, 1988). After growing to confluency the Schwann cells were washed free of these mitogens and cultured in DMEM supplemented with 10% fetal calf serum for 5 days.

Isolation of RNA and cDNA cloning

Isolation of total RNA and construction of a cDNA library from poly(A)$^+$ RNA of the distal segment of crushed sciatic nerves as well as selection of clones by differential colony hybridization using [^{32}P]dCTP-labelled first strand cDNA derived from both the distal part of crushed sciatic nerves (7 days after lesion) and non-injured sciatic nerve has been described previously (Spreyer *et al.*, 1990).

DNA sequencing and computer analysis

Subclones of cDNA inserts were obtained by Exonuclease III (ExoIII) deletion (Erase-a-base, Promega Biotec, Madison, WI) in pSP72/73 vectors. Sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using the T7 Sequencing Kit (Pharmacia-LKB). Sequence data were compared with the EMBL nucleotide sequence data library (release no. 25) and the SwissProt protein sequence databank (release no. 17) using the programs fstnscan, nmatpus, translate, fstpscan and pcompare (Intelligenetics, Mountain View, CA).

Northern blotting

Six to 10 μg of total RNA were fractionated on 1.4% agarose gels containing 15% formaldehyde and then transferred to Nytran NY13N membranes or nitrocellulose (Schleicher and Schuell). Prior to transfer, formaldehyde gels were incubated in 1.5 M NaCl/0.15 M Na-citrate at room temperature (RT) for 2 × 10 min. Nitrocellulose filters were prehybridized in 50% formamide, 5 × Denhardt's, 0.1% SDS, 5 × SSPE, 200 μg/ml sonicated salmon sperm DNA and 100 μg/ml poly(U) and hybridized in the same solution containing the nick translated probe (using [^{32}P]dCTP). For Nytran membranes the following prehybridization solution was used: 10% formamide, 200 mM NaCl, 50% dextran sulfate, 100 mM Tris, pH 7.5, 2% SDS and 0.1 mg/ml salmon sperm DNA. Prior to exposure the filters were washed in 2 × SSC/1% SDS at room temperature for 30 min and in 0.1 × SSC/1% SDS at 65°C for 15 min. Nytran filters were stained with methylene blue prior to hybridization.

In situ hybridization

Tissue sections were prepared from non-injured, transected and anastomosed nerves. Nerves were fixed by immersion in modified Bouin's solution (5% acetic acid, 4% paraformaldehyde, 0.8% picric acid). Segments of injured and non-lesioned control nerves were dissected, dehydrated, embedded in paraffin and cut into 4 μm sections. De-paraffinized sections were treated with proteinase K, acetylated and prehybridized at 55°C for 4 h according to Angerer *et al.* (1987).

Run-off transcripts from the 1.1 kb pCD25 cDNA fragment were prepared as described above using digoxygenin–UTP and the DIG–RNA labelling kit from Boehringer-Mannheim. After hybridization at 55°C overnight, RNase A treatment (10 μg/ml in 0.5 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 8) was carried out at 37°C for 20 min. The sections were then washed three times in 2 × SSC at 50°C for 20 min and three times in 0.1 × SSC at 50°C for 20 min. Digoxygenin detection was carried out following the manufacturer's instructions (Boehringer-Mannheim).

Immunohistochemistry

De-paraffinized sections were treated with 3% H_2O_2 in methanol for 5 min and then with 2% normal goat serum (NGS) in Tris buffered saline (TBS) for 30 min. A mouse monoclonal antibody to the 200 kDa neurofilament protein (clone RT97, 1:500, Boehringer-Mannheim) and rabbit antiserum to S100 protein (1:10 000, Sigma) diluted in 1% NGS/TBS were applied as primary antibodies at 5°C overnight. With periodic washes in TBS, sections were incubated with biotinylated secondary antibodies (1:200, Vector) for 30 min, then with avidin–biotinylated peroxidase complex (1:50, Vector) for 30 min and finally with the substrate diaminobenzidine/H_2O_2 for 5 min.

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References

Angerer,L.M., Stoler,M.H. and Angerer,R.C. (1987) In Valentino,K.L., Eberwine,J.H. and Barchas,J.D. (eds), *In Situ Hybridization. Application to Neurobiology.* Oxford University Press, NY, pp. 42–70.

Asbury,A.K. (1967) *J. Cell Biol.*, **34**, 735–743.

Bunge,R.P., Bunge,M.B. and Eldridge,C.F. (1986) *Annu. Rev. Neurosci.*, **9**, 305–328.

Chan,Y.L., Gutell,R., Noller,H.F. and Wool,I.G. (1984) *J. Biol. Chem.*, **259**, 224–230.

Ciccarelli,C., Philipson,L. and Sorrentino,V. (1990) *Mol. Cell. Biol.*, **10**, 1525–1529.

DiStefano,P.S. and Johnson,E.M. (1987) *J. Neurosci.*, **8**, 231–241.

Heumann,R., Lindholm,D., Bandtlow,C., Meyer,M., Radeke,M.J., Misko,T.P., Shooter,E.M. and Thoenen,H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8735–8739.

Ignatius,M.J., Gebicke-Härter,P.J., Skene,J.H.P., Schilling,J.W., Weisgraber,K.H., Mahley,R.W. and Shooter,E.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1125–1129.

Klein,P., Kanehisa,M. and DeLisi,C. (1985) *Biochim. Biophys. Acta*, **815**, 468–476.

Kozak,M. (1989) *J. Cell Biol.*, **108**, 229–241.

Lemke,G. (1988) *Neuron*, **1**, 535–543.

Lemke,G. and Chao,M. (1988) *Development*, **102**, 499–504.

Lindholm,D., Heumann,R., Meyer,M. and Thoenen,H. (1987) *Nature*, **330**, 658–659.

Malter,J.S. (1989) *Science*, **246**, 664–666.

Manfioletti,G., Ruaro,M.E., Del Sal,G., Philipson,L. and Schneider,C. (1990) *Mol. Cell. Biol.*, **10**, 2924–2930.

Meier,R., Spreyer,P., Ortmann,R., Harel,A. and Monard,D. (1989) *Nature*, **342**, 548–550.

Monuki,E.S., Weinmaster,G., Kuhn,R. and Lemke,G. (1989) *Neuron*, **3**, 783–789.

Monuki,E.S., Kuhn,R., Weinmaster,G., Trapp,B.D. and Lemke,G. (1990) *Science*, **249**, 1300–1303.

Müller,H.W., Ignatius,M.J., Hangen,D.H. and Shooter,E.M. (1986) *J. Cell Biol.*, **102**, 393–402.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.

Spencer,P.S., Politis,M.J., Pellegrino,R.G. and Weinberg,H.J. (1981) In Gorio,A., Millesi,H. and Mingrino,S. (eds), *Post-traumatic Peripheral Nerve Regeneration.* Raven Press, NY, pp. 411–426.

Spreyer,P., Schaal,H., Kuhn,G., Rothe,T., Unterbeck,A., Olek,K. and Müller,H.W. (1990) *EMBO J.*, **9**, 2479–2484.

Sunderland,S. (1978) *Nerves and Nerve Injuries*, 2nd edn. Churchill-Livingstone, Edinburgh.

Terry,L.C., Bray,G.M. and Aguayo,A.J. (1974) *Brain Res.*, **69**, 144–148.

Trapp,B.D., Hauer,P. and Lemke,G. (1988) *J. Neurosci.*, **8**, 3515–3521.
von Heine,G. (1984) *J. Mol. Biol.*, **173**, 243–251.

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Note added in proof

The nucleotide sequence data reported here will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession number X62431/rat cd25.