Laboratory Investigation

Lack of *NF1* expression in a sporadic schwannoma from a patient without neurofibromatosis

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Key words: neurofibromin, Schwann cells, tumor suppressor gene, neurofibromatosis

Summary

The neurofibromatosis type 1 (NF1) gene encodes a tumor suppressor protein, neurofibromin, which is expressed at high levels in Schwann cells and other adult tissues. Loss of *NF1* expression has been reported in Schwann cell tumors (neurofibrosarcomas) from patients with NF1 and its loss is associated with increased proliferation of these cells. In this report, we describe downregulation of *NF1* expression in a single spinal schwannoma from an individual without clinical features of neurofibromatosis type 1 or 2. Barely detectable expression of *NF1* RNA was found in this tumor by *in situ* hybridization using an *NF1*-specific riboprobe as well as by Northern blot and reverse-transcribed (RT)-PCR analysis. In Schwann cells cultured from this schwannoma, abundant expression of *NF1* RNA could be detected by Northern blot and RT-PCR analysis. These results suggest that, in some tumors, expression of *NF1* may be downregulated by factors produced within the tumor and may represent a novel mechanism for inactivating these growth suppressing genes and allowing for increased cell proliferation in tumors.

Introduction

Tumor suppressor genes (TSGs) are operationally defined by their altered expression in cancer cells [1]. Their absent or aberrant expression in tumors is hypothesized to result in loss of normal cell growth control, leading to the increased cell proliferation associated with progression to malignancy. The neurofibromatosis type 1 (NF1) gene is such a TSG whose expression is reduced in a wide variety of human cancers [2]. NF1 is an autosomal dominant disorder in which affected individuals manifest an increased incidence of tumor development [3]. These include benign tumors, such as optic pathway gliomas and neurofibromas, as well as malignant tu-

mors like neurofibrosarcomas, pheochromocytomas, rhabdomyosarcomas and leukemias [4].

NF1 encodes a large 250 kDa protein, neurofibromin, expressed in a wide variety of cell types including Schwann cells, oligodendrocytes, neurons and leukocytes [5, 6]. Since the hallmark of the disorder is the neurofibroma, a tumor composed predominantly of Schwann cells, initial studies have focused on the role of neurofibromin in Schwann cell growth control and demonstrated that neurofibromin expression increases during *in vitro* rat Schwann cell differentiation [7]. This result suggests that neurofibromin might play an important role in normal Schwann cell growth and differentiation such that its reduced expression in neurofibro-

mas or schwannomas might be associated with increased Schwann cell proliferation.

Examination of neurofibrosarcoma cell lines (malignant Schwann cell tumors derived from neurofibromas) from patients with NF1 have demonstrated altered *NF1* expression [8, 9]. Lack of neurofibromin expression correlated with increased levels of activated p21-ras, consistent with the function of neurofibromin as a GTPase-activating protein for p21-ras. As part of an ongoing study to examine the expression of neurofibromin in human Schwann cell tumors, we found one patient without clinical features of NF1 or NF2 (bilateral vestibular neurofibromatosis) who presented with a spinal schwannoma lacking expression of *NF1*. In this report, we demonstrate that the Schwann cells cultured from this tumor, unlike the tumor itself, have abundant *NF1* expression. These results suggest that *NF1* expression might be downregulated in selected tumors through mechanisms other than mutation and offer the possibility that neurofibromin expression may be regulatable in selected tumors.

Materials and methods

Tumors

Fresh surgical tissue from the spinal schwannoma was obtained in the operating room according to the guidelines established by the Washington University Institutional Review Board (IRB protocol # 93-0725) and divided into three portions. One portion was wrapped in aluminum foil and immediately snap-frozen in liquid nitrogen and stored at −70° C. Another portion was immersed in fresh 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 hours at 4° C prior to processing for *in situ* hybridization and immunohistochemistry. The third portion was placed in Dulbecco's Modified Essential Medium (DMEM) containing 10% fetal bovine serum (Gibco-BRL) and 1% pencillin/streptomycin for culturing of Schwann cells and fibroblasts.

Culturing of tumor Schwann cells

Cells were enzymatically dissociated from tumor tissue or adult human peripheral nerve and attached to glass coverslips or 10 cm plastic dishes coated with poly-L-lysine as previously described [10]. Fibroblasts were purified by serial subculture on uncoated plastic in basal feeding medium consisting of Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 200 U/ml penicillin and 0.2 mg/ml streptomycin. After four successive passages, the cultures contained > 98% fibroblasts as determined by indirect immunofluorescent staining for a fibroblast surface protein [11]. Schwann cells were propagated in basal feeding medium supplemented with conditioned medium from a transfected CHO cell line secreting recombinant human glial growth factor [12] and 0.5 mM forskolin and 0.5 mM isobutylmethylxanthine to suppress fibroblast growth (JLR, manuscript in preparation). These cultures contained predominantly Schwann cells which were identified by indirect immunofluorescent staining for S-100 protein expression [10].

Riboprobe production

A cDNA containing nucleotides 8237 to 8457 of *NF1* [13] was cloned into pBlueScript II. KS (−). To synthesize antisense and sense riboprobes, this plasmid was linearized with the appropriate restriction endonucleases (Sal 1 for sense and Xba 1 for antisense) and transcribed *in vitro* in the presence of 90 μCi [^{35}S]UTP (Amersham) using either T7 (sense) or T3 (antisense) RNA polymerases.

In situ hybridization

The *in situ* hybridization procedures employed in our laboratory follow the method described elsewhere [14]. Briefly, individual sections were hybridized overnight at 55–60° C with either antisense or sense riboprobes (1×10^6 cpm/slide) diluted in a 60 microliters of hybridization mixture containing 50% deionized formamide, 10% dextran sulfate, 1 ×

Denhardt's, 4 × SSC, 10 mM dithiothreitol, 1 mg/ml yeast tRNA, and 1 mg/ml denatured salmon sperm DNA. The following day, the sections were rinsed twice in 4 × SSC for 30 min at 37° C, treated with RNase A (40 µg/ml) for 30 min at 45° C and then rinsed at 37° C in 2 × SSC (2 × 20 min); 0.5 × SSC (2 × 20 min), and 0.1 × SSC (30 min). The sections were then briefly dipped in distilled water, followed by 95% ethanol, and air dried. Sections were exposed to BioMax film (Kodak) for 3 days to generate film autoradiograms, and then dipped in Kodak NTB-2 liquid emulsion and stored in dessicated light-tight boxes at 4° C for 10–15 days. Slides were developed in Kodak D19 and fixed in Kodak Fixer, rinsed in distilled water, counterstained with hematoxylin and eosin, dehydrated, and coverslipped with 50% DPX.

Control experiments were performed to assess the specificity of the riboprobes. Sections were incubated with individual sense strand ^{35}S-labeled riboprobes or were pretreated with RNase A (Boehringer Mannheim; 20 µg/ml for 30 min at 37° C) followed by hybridization with individual antisense riboprobes. In each case, control hybridizations resulted in complete loss of hybridization signal. Sections were viewed using darkfield microscopy, and all images were acquired on a Nikon Microphot FXA microscope utilizing a Kodak DCS 2000 digital camera and Adobe Photoshop 2.5.1 software for Macintosh.

Antibodies

The B3A.1 rabbit polyclonal anti-neurofibromin antibody was prepared against a fusion protein containing the terminal 73 amino acids of the full length neurofibromin protein (excluding the 48a exon coding sequence) as previously described [15]. S-100-β antibodies were purchased from Boehringer Mannheim and used according to the manufacturers recommendations.

Immunohistochemistry

Paraffin-embedded sections were surrounded with a Teflon coating using a Pap-pen (Kyota Co.), and incubated for one hour in a blocking solution consisting of Superblock buffer (Pierce) 0.3% Triton X-100 and 0.3% Tween-20, 1.0% porcine gelatin (Sigma) and 1.5% normal goat serum (Vector). Primary and secondary antibodies were diluted in the same solution diluted 1:1 with Superblock buffer. Primary incubations were placed at 4° C overnight (12–16 hours). B3A.1 was used at a 1:300 dilution while the S-100 antibody was used at a 1:200 dilution. Slides were then washed 3 × 5 minutes with phosphate-buffered saline, pH 7.2 (PBS) and placed in secondary antibody (anti-rabbit Cy3 conjugated antibody; Jackson Immunochemicals), washed 3 × 5 minutes in PBS and coverslipped in PBS, for examination under fluorescence.

Northern blot

Total RNA was isolated from cells after rinsing the dishes in cold PBS using a modification of the acid-phenol method (RNAzol B; Tel-Test, Inc). RNA concentrations were calculated by UV absorption and stored at − 70° C in DEPC-treated water. Samples of total RNA (20 µg/lane) were separated on 1.2% agarose-0.37 M formaldehyde gels for 3.5 hours, stained briefly with ethidium bromide and photographed. RNA was transferred onto nitrocellulose membranes (Hybond, Amersham) and cross-linked by UV exposure [16]. The 1.2 kb EcoRI fragment of plasmid P5, 2.2 kb EcoR1 fragment containing full-length *NF2* ([17]; kindly provided by Dr. J. Gusella, MGH), 1.0 kb Po cDNA fragment, and 1.0 kb β-actin cDNA probe were labeled with ^{32}P-dCTP using random hexamers and purified by spin-column chromatography. Membranes were prehybridized in 1× Denhardt's solution (1% SDS/0.2 M dextran sulfate/1 M NaCl) containing 100 µg per ml salmon sperm DNA for 3–12 hours at 65° C, and then hybridized with the probe overnight at 65° C. Hybridizations with the *NF2* cDNA probe were performed at 42° C overnight. Membranes were then washed in 2 × SSC, 0.1% SDS and exposed to film at − 70° C for 2–5 days.
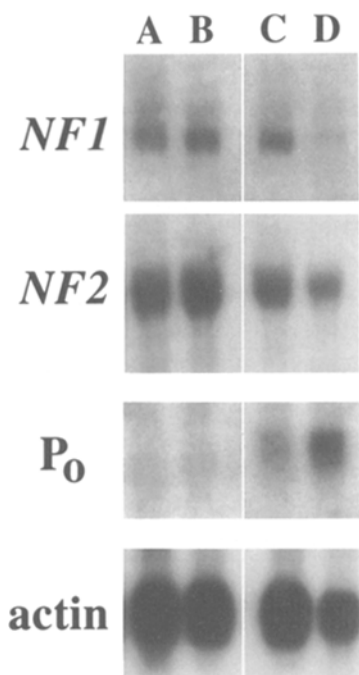
Fig. 1. Expression of NF1 *RNA in tumor 021194 and cultured Schwann cells.* Total RNA extracted from normal fibroblasts (lane A), normal Schwann cells (lane B), 021194 Schwann cells (lane C), and tumor 021194 (lane D) were analyzed by Northern blot analysis as described in the Materials and Methods section. The Hybond-N filter containing these RNAs was sequentially probed with the P5 *NF1* cDNA probe, the complete *NF2* cDNA probe (pBlueScript.JJR.1), a Po cDNA probe and the β-actin probe. Barely detectable levels of *NF1* RNA could be seen in the 021194 tumor whereas abundant *NF1* RNA was found in the Schwann cells cultured from this tumor. Equivalent amounts of *NF2* RNA were seen in the tumor relative to β-actin expression. Normal cultured Schwann cells express equivalent amounts of *NF1* mRNA as cultured fibroblasts (data not shown).

Reverse-transcribed PCR analysis

RNA was extracted by conventional methods and three micrograms were reverse-transcribed as previously described using SuperScript II (Bethesda Research Laboratories) and RNase inhibitors [7]. *NF1* cDNA was amplified using primers (5'-ATT GTGATCACATCCTCTGATTGG-3' and 5'-CA TGATTGGCAATACTCTGAA-3') which generate both the type 1 and type 2 *NF1* isoform products for 25 cycles with a 55 degree annealing step [18,19]. Type 1 *NF1*, lacking the 63 nucleotide exon 23a insertion, and type 2 *NF1* containing this insertion, migrate as 376 and 439 nucleotide products, respec-

tively. S-100-β primers amplify a 223 nucleotide product (5'-GGGAATTCGGATGTCTGAGC TGGAGAAG-3' and 5'-GCGGATCCACTCCT GGAAGTCACACTCC-3'). Cyclophilin primers amplify a 206 nucleotide product used as an internal control for the amount of RNA in each sample (5'-ATGGTCAACCCCACCGTGTT-3' and 5'-CG TTGTAAGTCACCACCCT-3'). Twenty-five cycles were chosen based on previous experiments demonstrating a linear relationship between input DNA and obtained RT-PCR product with these conditions. RT-PCR products were separated on 8% polyacrylamide gels along with φχ-Hae III markers and visualized by staining with ethidium bromide.

Results

Patient 021194

C.S. is an eleven year old body first noted to have thoracic spinal schwannomas at age 6 when he presented with back pain, lower extremity hyper-reflexia and mild leg weakness. Surgical resection of this tumor confirmed the diagnosis of schwannoma. Over the next few years, he underwent two additional surgical procedures for tumor debulking, most recently in February 1994 when he was seen in our Neurofibromatosis Program. At that time, he had four cafe-au-lait spots, no Lisch nodules, no family history of neurofibromatosis, a normal head MRI (no vestibular neuromas or meningiomas), normal stature and head circumference for his age (50th percentile) and no axillary or inguinal freckling. Mild scoliosis was noted on examination (ten degrees with a convexity to the right). He has continued to do well after his surgeries with only minimal lower extremity weakness.

Examination of the tumor resected in 1994 demonstrated eosinophilic spindle cells with prominent nucleolar palisading and Verocay body formation. The tumor consisted of densely cellular Antoni A tissue with relatively little Antoni B tissue. Immunohistochemical analysis with S-100 antibodies demonstrated intense labeling of the Schwann cells in the tumor.
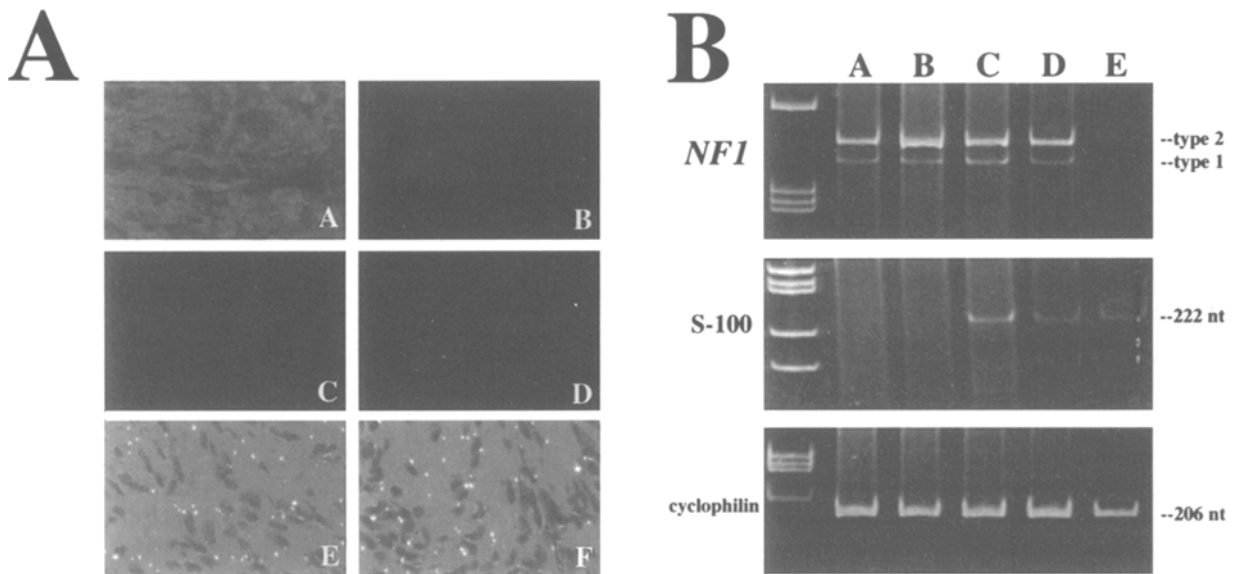
Fig. 2. Expression of NF1 in tumor 021194. (A) NF1 expression by in situ hybridization and immunohistochemistry. Abundant expression of S-100 protein could be detected in the tumor (panel A) whereas no neurofibromin was found using the B3A.1 antibody (panel C). Preimmune control for the B3A.1 antibody (panel D) and secondary antibody alone control (panel B) demonstrated no staining of the tumor. Likewise, no NF1 RNA expression could be detected by in situ hybridization as described in the Material and Methods section. Panel E depicts hybridization with the sense NF1 cRNA probe while antisense NF1 cRNA probe hybridization is shown in panel F. No differences between sense and antisense hybridization could be demonstrated. (B) Reverse-transcribed (RT) PCR analysis of tumor 021194 and derivative Schwann cells and fibroblasts. Three micrograms of RNA was reverse-transcribed as described in the Materials and Methods section and used for PCR analysis of NF1, S-100 and cyclophilin RNA expression. The conditions and cycle number were chosen based on previous experiments demonstrating a semi-quantitative relationship between input cDNA and output PCR product. Whereas abundant amounts of cyclophilin and S-100 RNA could be detected in tumor 021194, little NF1 RNA could be detected (lane E). In contrast, NF1 RNA could be detected in normal Schwann cells (lane C) and fibroblasts (lane A) as well as the cultured Schwann cells (lane D) and fibroblasts (lane B) from this tumor.

Lack of NF1 expression in Schwannoma 021194

A representative Northern blot is shown in Fig. 1. As can be seen, there is abundant expression of NF2, myelin Po and actin mRNA in the tumor (lane D) but markedly reduced expression of NF1. Abundant expression of NF1, NF2, actin and myelin Po RNA could be detected in Schwann cells derived from normal sciatic nerve (data not shown). Likewise, no significant NF1 expression could be detected by in situ hybridization using an antisense NF1 cDNA riboprobe (Fig. 2A; panel E) when compared to the NF1 sense riboprobe control (Fig. 2A; panel F). This result was verified by immunohistochemistry using B3A.1 polyclonal neurofibromin antiserum (Fig. 2A; panel C). Positive antisense NF1 riboprobe and B3A.1 antibody staining were observed with other tumors and rat brain controls

(data not shown). In contrast, the tumor expresses S-100 protein as detected using the S-100 antibody (Fig. 2A; panel A). Appropriate negative controls included preimmune B3A.1 antiserum (Fig. 2A; panel D) and secondary antibody alone (Fig. 2A; panel B). Lastly, the expression of NF1 mRNA was nearly undetectable by RT-PCR analysis whereas S-100 and cyclophilin mRNA could be easily demonstrated (Fig. 2B; lane E). These results collectively demonstrate that NF1 expression is greatly reduced in this Schwann cell tumor.

Cultured Schwann cells from tumor 021194 demonstrate NF1 expression

Schwann cells cultured from this tumor were found to express NF1 mRNA by Northern blot and RT-
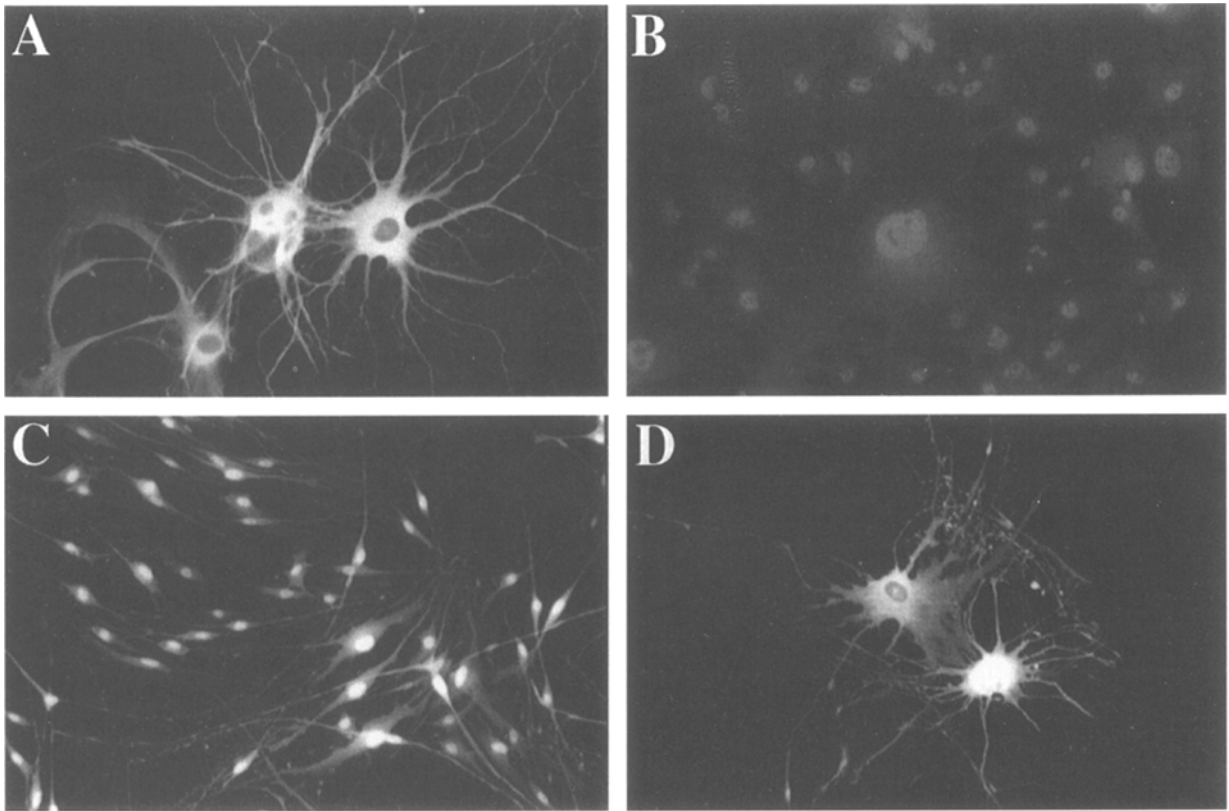
Fig. 3. Cultured Schwann cells from tumor 021194 and from normal human peripheral nerve. Cells seeded on glass coverslips were stained for S-100 protein by indirect immunofluorescence. Approximately 30% of the cells in primary dissociates of the tumor stained intensely with antibodies to S-100 (A). Morphologically, these cells differed distinctly from primary Schwann cells isolated from normal nerves which are typically bipolar (C). Early passage Schwann cells from the tumor resembled the enlarged cells with multiple processes which began to appear in long-term cultures of normal Schwann cells shown here at passage 12 (D). The number of multiprocess Schwann cells cultured from the tumor declined with successive passages. By passage five, more transformed-appearing cells expressing low levels of S-100 in the cytoplasm and nucleus predominated (B). Magnification is approximately 100 ×.

PCR analysis. High levels of *NF1* expression can be seen in the cultured Schwann cells from this tumor by Northern blot (Fig. 1; lane C) and RT-PCR (Fig. 2B; lane D). Likewise, abundant expression of S-100, *NF2,* actin and Po mRNA could be demonstrated in these Schwann cells (Fig. 1; lane C and Fig.2B; lane D) as compared to Schwann cells obtained from normal human sciatic nerve (Fig. 1; lane B and Fig. 2B; lane C). Immunocytochemistry using the B3A.1 polyclonal antibody also detected neurofibromin expression in these cultured cells (data not shown). These results demonstrate that *NF1* expression may be upregulated in cultured Schwann cells taken from a Schwann cell tumor in which no *NF1* expression could be detected.

Tumor 021194 Schwann cells maintain NF1 expression in culture

The expression of *NF1* in Schwann cells taken from a tumor not expressing detectable levels of *NF1* mRNA or protein may represent an artifact of *in vitro* manipulation. Two possibilities exist to explain the above results: (1) normal Schwann cells contaminating the tumor were expanded in lieu of the schwannoma tumor Schwann cells and (2) growth factors added to the cultured cells induced the expression of *NF1.* To address these possibilities, additional analysis was performed on these cultured Schwann cells. First, these tumor Schwann cells were morphologically different than 'normal' Schwann cells (Fig. 3). Second, these Schwann cells

retained their ability to express *NF1* mRNA even
after three months in culture as demonstrated by
RT-PCR (data not shown). Lastly, removal of all ex-
ogenously added growth factors from the medium
had no effect on *NF1* expression, in that Schwann
cells grown in the presence or absence of these fac-
tors express similar amounts of *NF1* mRNA by RT-
PCR (Fig. 4).

Discussion

NF1 and NF2 are distinct clinical entities which
have in common tumors of Schwann cell origin. Pa-
tients with NF1 present with cafe-au-lait spots,
freckling in non-sun-exposed areas, Lisch nodules
(iris hamartomas) and neurofibromas (benign tu-
mors composed predominantly of Schwann cells)
[20]. NF2, on the other hand, more commonly pre-
sents with bilateral vestibular neuromas and menin-
giomas without Lisch nodules, freckling or cafe-au-
lait spots [21]. In both disorders, there is an in-
creased incidence of malignancy, owing to the fact
that both genes are tumor suppressor genes.

Examination of *NF1* expression in NF1-related
Schwann cell tumors has demonstrated loss of neu-
rofibromin expression in malignant neurofibrosar-
coma cell lines [8, 9]. Introduction of a portion of
NF1 into these malignant Schwann cells resulted in
decreased cell proliferation, suggesting that the
lack of neurofibromin downregulation of p21-ras
activity is associated with tumor cell proliferation.
However, less is known about the expression of *NF1*
in the benign neurofibroma. Recent evidence from
our laboratory suggests that *NF1* expression is re-
duced in the Schwann cells, but not in the fibro-
blasts, cultured from NF1 neurofibromas (JLR and
DHG, manuscript in preparation). Studies examin-
ing the expression of *NF2* in Schwann cell tumors
have demonstrated mutations in over fifty percent
of NF2-related as well as sporadic vestibular neuro-
mas [22, 23]. Other studies have shown frequent de-
letions and loss of heterozygosity in the region of
NF2 in both sporadic and NF2-related vestibular
neuromas [24, 25].

The results described in this manuscript demon-
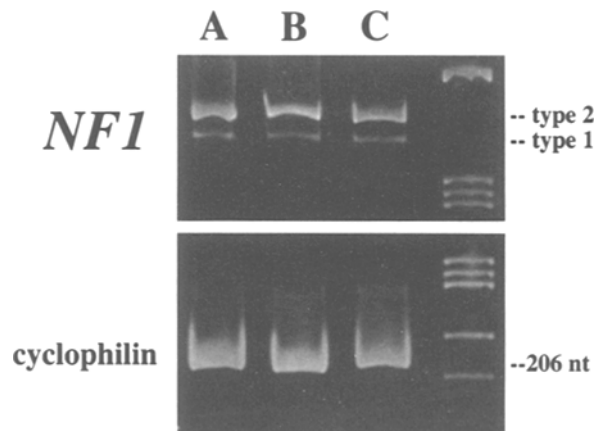strate reduced expression of *NF1* mRNA and pro-

Fig. 4. NF1 *RNA expression is detected in cultured tumor Schwann cells growth in the presence or absence of growth factors.* Three micrograms of RNA was reverse-transcribed as described in the Materials and Methods section and used for PCR analysis of *NF1* and cyclophilin RNA expression. The conditions and cy-cle number were chosen based on previous experiments demon-strating a semi-quantitative relationship between input cDNA and output PCR product. There is continued expression of *NF1* RNA in these cultured cells grown in the presence (lane B) or absence (lane C) of growth factors (recombinant glial growth factor and forskolin) compared to the original batch of 021194 tumor Schwann cells taken after three weeks *in vitro* (lane A).

tein in a Schwann cell tumor from a patient without
NF1 or NF2. This finding is not surprising given the
possible function(s) of neurofibromin in Schwann
cell differentiation. The unexpected result of 'nor-
mal' *NF1* expression in the cultured Schwann cells
from this tumor suggest a novel mechanism for
growth regulation in tumors. The lack of *NF1* ex-
pression in the tumor is unlikely to be artifactual
since normal expression of other mRNAs (S-100,
NF2, Po and actin) could be demonstrated using a
variety of different molecular biology techniques
including Northern blot, RT-PCR, *in situ* hybridiza-
tion and immunohistochemistry. Similarly, the ex-
pression of *NF1* mRNA and protein in the cultured
Schwann cells is unlikely to be artifactual. Two ma-
jor possibilities can be invoked to explain this in-
creased expression: *(1) The increased expression is
related to the culture conditions.* To this end, we have
demonstrated that the expression of *NF1* mRNA is
not dependent on exogenously added growth fac-
tors, as removal of these factors does not result in
decreased *NF1* expression. Likewise, there is no
change in *NF1* expression in these cells over time in

culture and the 021194 tumor cells still express *NF1* mRNA after 3 months in culture. Even if the act of plating these cells *in vitro* resulted in increased *NF1* expression, this would suggest that at least one functional *NF1* allele exists in these tumor cells which is responsive to upregulation. *(2) The increased expression of NF1* mRNA is related to the selective expansion of contaminating 'normal' Schwann cells. This is an unlikely possibility since the morphological and growth characteristics of these Schwann cells do not resemble those seen in cultured Schwann cells from normal sciatic nerve. In addition, there is no growth advantage for normal Schwann cells that would contribute to their preferential expansion. On the contrary, the 'tumor' Schwann cells would be expected to be preferentially expanded since their growth potential is augmented by definition during the process of malignant transformation. Studies are underway to determine whether *NF1* expression can be downregulated by implanting the 021194 Schwann cells into mice.

Examination of other Schwann cell tumors in our laboratory has failed to detect further examples of reversible *NF1* downregulation. Although this is not the common mechanism for reducing TSG expression in tumors, it represents an important observation in that it offers exciting possibilities for the potential treatment of *selected* tumors through modulation of *NF1* expression. Future studies directed at defining the mechanism(s) underlying transcriptional regulation of *NF1* expression in normal and tumor cells may provide potential therapies for the pharmacologic treatment of these tumors.

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