DEVELOPMENTAL BIOLOGY 161, 538-551 (1994)

Differential Expression and Tissue Distribution of Type I and Type II Neurofibromins during Mouse Fetal Development

DUONG P. HUYNH, TAMILLA NECHIPORUK, AND STEFAN M. PULST[1]

Neurogenetics Laboratory and Division of Neurology, Cedars-Sinai Research Institute, University of California, Los Angeles, California 90048

Accepted October 25, 1993

Mutations in the NF1 gene may cause developmental abnormalities and the formation of a variety of tumors of neural crest origin in humans. The NF1 gene codes for a large protein, neurofibromin (nf), which is structurally and functionally related to yeast and human ras-GTPase-activating proteins (ras-GAPs). Recently, two transcripts coding for type I and type II nf with different ras-GAP activity have been identified. Since ras proteins do not appear to be significantly regulated during mouse development, we examined if differential expression of neurofibromins may provide evidence for a role of nfs in regulating ras-mediated cell proliferation and differentiation. Nfs were expressed as early as E8. At E11 a marked increase of NF1 transcripts occurred and was associated with expression of nfs in all tissues. Type I and type II nfs each showed a different time course of expression and tissue localization, with type II nf present mainly from E8 through E10, although in the heart type II nf was present at E12. In some tissues such as heart and dorsal root ganglia rapid increases and decreases of nfs were detected related to differentiation of these tissues. These results are consistent with a role of nfs in regulating ras-mediated cell proliferation and differentiation during development and support distinct functional roles for type I and type II nfs.

INTRODUCTION

Von Recklinghausen neurofibromatosis (NF1) is one of the most common Mendelian diseases, affecting 1 in 3500 individuals. NF1 affects the development and growth control of many tissues, but especially those derived from the neural crest (reviewed in Pulst, 1991). Common manifestations are benign tumors of the peripheral nerves comprised mainly of Schwann cells (neurofibromas), tumors of the iris (Lisch nodules), and hyperpigmentation of melanocytes (café au lait spots) (Riccardi, 1981). Patients with NF1 are at an increased risk for specific kinds of malignancies, including neurofibrosarcomas, astrocytomas, pheochromocytomas, and embryonic rhabdomyosarcomas (Bader, 1986; Korf, 1990). In addition, developmental abnormalities are common, such as learning disabilities and presumed hamartomatous changes within the brain on magnetic resonance imaging (Shu *et al.*, 1993) as well as typical bony changes such as deformities of the vertebral bodies and long bones.

The gene for NF1 has been cloned (Cawthon *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990). Its coding sequence contains a large region of homology with the products of the yeast IRA1 and IRA2 genes (Buchberg *et al.*, 1990) and a smaller region of homology with the mammalian GTPase activating protein (GAP) (Xu *et al.*, 1990a,b). The complete cDNA encodes a hydrophilic protein of 2818 amino acids (Marchuk *et al.*, 1991) which is now called neurofibromin. Neurofibromin shows a very high degree of sequence conservation in different species. For example, the amino acid sequences of human and murine neurofibromin domains vary by only 1% (Buchberg *et al.*, 1990; Kyritsis *et al.*, 1992).

IRA1 and IRA2 as well as mammalian GAP function as negative regulators of ras, but may also serve as target effectors for ras proteins (reviewed in Barbacid, 1987; and Hunter, 1991). Expression studies of the NF1-GAP-related domain (NF1-GRD) confirmed that this domain interacted with ras p21 proteins, and subsequently stimulated the hydrolysis of GTP-$p21^{ras}$ (Golubic *et al.*, 1991; Han *et al.*, 1991; Martin *et al.*, 1990; Xu *et al.*, 1990a). Studies with cell lines derived from neurofibrosarcomas (Basu *et al.*, 1992; DeClue *et al.*, 1992) of NF1 patients also showed that these cells produced lower amounts of neurofibromin than comparable normal cells and that the produced neurofibromin was not efficient in catalyzing the hydrolysis of GTP-$p21^{ras}$ proteins. These observations suggested that the NF1 gene, at least in part, functions as a tumor suppressor gene.

[1] To whom correspondence should be addressed at the Division of Neurology, Room 8909, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, California 90048.

0012-1606/94 $6.00

Since these studies involved only the proposed catalytic domain of neurofibromin, they did not exclude the possibility that full-length neurofibromins may have other functions independent of ras proteins.

Additional NF1 transcripts have been identified (Marchuk *et al.*, 1991). The type II transcript consists of a 63-basepair (bp) insert coding for a 21-amino-acid sequence in the conserved region of the GAP-related domain (Nishi *et al.*, 1991; Suzuki *et al.*, 1991). The type III transcript, on the other hand, contains an additional 54-bp coding for an 18-amino-acid insert near the C-terminus of the protein (Cawthon *et al.*, 1990). Since the type II transcript encodes an altered GAP-related domain, much interest has focused on the function of type II neurofibromin. Several lines of evidence have indicated that type II neurofibromin may have significant functional roles. First, the 21-amino-acid (aa) insert of type II neurofibromin is evolutionarily conserved across species (Andersen *et al.*, 1993). Second, expression studies with the NF1-GRD region showed that both type I and II neurofibromins were capable of stimulating the hydrolysis of GTP-$p21^{ras}$, although type II neurofibromin had less catalytic efficiency and a lower sensitivity to lipid inhibition (Andersen *et al.*, 1993; Uchida *et al.*, 1992). However, since these studies were performed with a short region of NF1-GRD, different catalytic activities may be obtained with full-length neurofibromin isoforms.

Despite the importance of ras proteins in regulating cellular proliferation and differentiation (Barbacid, 1987; Hunter, 1991), their expression does not appear to be developmentally regulated. Almost constant levels of c-Ha-ras, c-Ki-ras, and N-ras mRNA transcripts in whole mouse homogenates were observed throughout murine development from E8 to E16 (Leon *et al.*, 1987; Muller *et al.*, 1982, 1983; Slamon and Cline, 1984). These observations suggest that other proteins regulate ras activity.

Although human NF1 mutations cause developmental abnormalities, little is known about the developmental pattern of NF1 gene expression and neurofibromin-mediated regulation of ras activity during development. In 4- to 14-day postnatal mice, an NF1 transcript of 13 kb was detected by Northern blot analysis (Buchberg *et al.*, 1990). The highest level of expression was seen in brain (Buchberg *et al.*, 1990). No data regarding expression of different neurofibromin transcripts during mouse development are available and studies using human fetal tissue have been incomplete and conflicting (Nishi *et al.*, 1991; Suzuki *et al.*, 1991).

Recently, we and others described antisera directed against different regions of neurofibromin (Basu *et al.*, 1992; Daston *et al.*, 1992; DeClue *et al.*, 1991; Gutmann *et al.*, 1991; Hattori *et al.*, 1992; Huynh *et al.*, 1992). These studies estimated a neurofibromin of an apparent molecular weight of 250–350 kDa. Western blots of murine tissues showed that neurofibromin was enriched in adult mouse and rat brain with only limited amounts detected in nonneural tissues (Golubíc *et al.*, 1992; Hattori *et al.*, 1992; Daston *et al.*, 1992).

Here we report the developmental expression of neurofibromins at the level of NF1 mRNAs and proteins in mouse embryos. Furthermore, we characterize the abundance and distribution of type I and type II neurofibromins by reverse PCR and immunohistochemical methods. Although neurofibromin expression in *adult* mice is greatly enriched in the nervous system, it was present at high levels in nonneural embryonic tissues. There was a marked increase of both NF1 transcript and gene product between Embryonic Days E10 and E11. Type II neurofibromin appears to be the predominant molecular species in neural tissues from E8 through E10 and in E12 cardiac tissue.

METHODS

RNA isolation and Northern blots. Developing mice (NIH-Swiss) of gestation ages 8 to 16 days were purchased from Harlam BioProducts for Science. Total RNA was isolated by the single-step method (Chomczynski and Sacci, 1987). Five hundred milligrams of whole murine fetus was homogenized in 5 ml of TRI-SOLV buffer (Biotecx Laboratories, Inc.), and RNA was purified according to the manufacturer's protocol (Biotecx Bulletin No. 22, 1992).

Twenty micrograms of total RNA from each sample was resolved in a 1.2% formaldehyde agarose gel (Ausubel *et al.*, 1989). The RNA was then transferred to a GeneScreen Plus membrane (Dupont) as follows. The gel was first rinsed with RNase-free water four times for 5 min each to remove formaldehyde, then equilibrated in 10× SSC (1× = 150 m*M* NaCl, 150 m*M* Na-citrate, pH 7.0) for 50 min. The RNA was then capillary-transferred to a prewetted GeneScreen Plus membrane for 24 hr. The RNA bound membrane was then rinsed in 2× SSC two times to remove gel debris, air-dried, uv-fixed (Stratagene, CA), and baked for 2 hr at 80°C.

The membrane was prehybridized for 2 hr at 60°C with 10 ml of hybridization buffer (10% dextran sulfate, 1% SDS, 1 *M* NaCl, 100 μg/ml salmon sperm DNA) in a hybridization incubator (Robin Scientific). After prehybridization, denatured ^{32}P-labeled cDNA insert (10 ng/ml) was added directly into the hybridization tube. The hybridization was allowed to continue for 24 hr at 60°C.

The membrane was then washed two times with 2× SSC for 5 min each at room temperature, two times with 2× SSC/1% SDS for 30 min each at 60°C, and once with 0.1× SSC for 30 min at room temperature.

cDNA synthesis and PCR analyses. The cDNA synthesis was performed using the Promega Reverse Transcription System. Two micrograms of total RNA was denatured at 70°C for 5 minutes and then reverse-transcribed at 42°C for 15 min in a 20-μl reaction mixture consisting of 10 m*M* Tris–HCl, pH 8.8, 50 m*M* KCl, 5 m*M* $MgCl_2$, 0.1% Triton X-100, 1 m*M* each dNTP, 20 units rRNasin ribonuclease inhibitor, 15 units AMV reverse transcriptase, 0.5 μg oligo$(dT)_{15}$ primer. The transcription reaction was terminated by boiling for 5 min followed by incubation in ice-water bath for at least 5 min.

Two synthetic oligonucleotide primers for PCR were designed according to Suzuki *et al.* (1991) with modification for the rat NF1 sequence (Kyritsis *et al.*, 1992; GCG Accession No. M82826). The sense primer was NFS2R, GAGTTGGCAGACTCCATGCA (2794/2813) and the antisense primer was NFA1, ATCCCT]chGCTTCATACGGTGA (complementary to 3193/3212). Two microliters of the cDNA reaction mixture was added to a PCR reaction mixture (total 10 μl) containing 80 ng primer NFS2R, 0.4 ng ^{32}P end-labeled NFA1 primer, 250 μM dNTP, 50 m*M* KCl, 10 m*M* Tris, pH 9.0, 0.1% Triton X-100, 1.5 m*M* $MgCl^2$, and 1.25 units of *Taq* DNA polymerase. Thirty-five PCR reaction cycles were carried out. Each cycle consisted of 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min. After the PCR reaction, a 5-μl aliquot was added to 20 μl of formamide dye (96% formamide, 10 m*M* EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), and 5 μl was loaded to a 5% polyacrylamide gel with 7 *M* urea (SequaGel/National Diagnostics). The DNA was fractionated at 160 V for 3 hr. The gel was exposed to Kodak X-OMAT film at −70°C for 15 to 30 min and developed by an automatic X-ray film developer. Bands were quantitated using a laser densitometer (Helena Laboratories) as described previously (Korenberg *et al.*, 1987).

Immunohistochemistry. Midsagittal paraffin-fixed 7-μm sections of mouse embryos were purchased from Novagen (Madison, WI). Paraffin was removed by four 5-min washes in xylene followed by three 5-min washes in 100% ethanol, three 5-min washes in 95% ethanol, three 5-min washes in 70% ethanol, three 5-min washes in 50% ethanol, and two 5-min washes in 100 m*M* PBS, pH 7.5.

The rehydrated sections were treated (as described in Huynh *et al.*, 1992) with Endo/Blocker (Biomeda) to inhibit endogenous peroxidase followed by 5 min in Auto/Zyme (Biomeda) at 37°C to release overfixed proteins for antibody accessibility. To block nonspecific antibody binding sites, the sections were treated with a solution containing 3% normal goat serum, 0.1% Triton X-100, 0.05% Na-azide, 100 m*M* PBS, pH 7.4) for 20 min followed by avidin/biotin blocker for 20 min. After each nonspecific blocking step, the sections were rinsed three times with 100 m*M* PBS, pH 7.4.

Sections were incubated with 1/100 dilution (16 ng/ml) of affinity-purified NF1C or GAP4 antibodies (ABs) for 2 hr at room temperature. [NF1C peptide = KAPKROEMESGITT, residues 2531–2544; GAP4 peptide = CHSLLNKATVKEKKKNKKS of the 21-aa insert]. Sections were then washed three times with 100 m*M* PBS. The primary antibody was detected using the Vector ABC elite Peroxidase kit. Prior to peroxidase color development, sections were briefly exposed to Peroxidase Enhancer (Biomeda).

Both GAP4 and NF1C antibodies were affinity-purified using their respective peptide coupled to a C4 Sepharose as previously described (Pulst *et al.*, 1986).

Western blot analyses of murine tissues. Western blot was performed according to Towbin *et al.* (1979) with the following modifications. Rodent brains were homogenized in lysis buffer (100 m*M* Tris, pH 8.0, 150 m*M* NaCl, 1% Triton X-100, 5 U/ml of phenylmethanesulfonyl fluoride (PMSF), 2 U/ml aprotinin). The proteins were resolved on a 4–20% SDS-polyacrylamide gradient gel (Bio-Rad). The fractionated proteins were transferred to a nitrocellulose filter (Towbin *et al.*, 1979). The filter was rinsed briefly with TBS (150 m*M* NaCl, 50 m*M* Tris, pH 8.0), blocked with 5% nonfatal dry milk, and then incubated with indicated affinity-purified antibodies at a dilution of 1/1000–1/2000 overnight at 4°C. The primary antibody was detected using a Bio-Rad Immuno-Blot Assay kit for alkaline phosphatase-conjugated anti-rabbit IgG.

To test for the specificity of the antibody, peptide used to raise the indicated antibody was co-incubated with the antibody at a concentration of 100 μM overnight at 4°C. The preabsorbed antibody was then diluted to the desired dilution for the staining of Western blots and tissue sections.

RESULTS

Expression of Neurofibromin mRNA in Developmental Mice

To investigate the relative expression of neurofibromin mRNA in murine fetuses, we isolated total RNA from fetal mice at stages E8 to E16 and performed RNA blotting using pFB8 as an NF1 cDNA probe. The pFB8 cDNA clone contains a 2068-bp insert of the 3′-end of the human neurofibromin gene (Xu *et al.*, 1990a) and thus detects all three known NF1 transcripts. Figure 1A shows an autoradiograph of a Northern blot of total RNA isolated from entire mouse fetuses. A 13.5-kb mRNA band was detected in all development stages; no other transcripts were seen (data not shown). In order to normalize the amount of NF1 transcripts, Northern

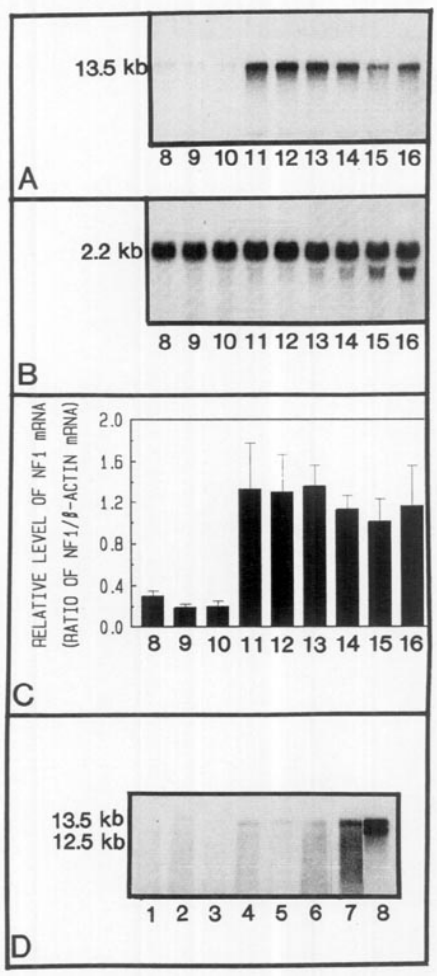

FIG. 1. Expression of neurofibromin mRNA in developing and adult mice. (A) Autoradiograph of a Northern blot probed with neurofibromin cDNA (pFB8). Each lane contains 20 μg of total RNA from mice of gestational age E8 (lane 8), E9 (lane 9), E10 (lane 10), E11 (lane 11), E12 (lane 12), E13 (lane 13), E14 (lane 14), E15 (lane 15), and E16 (lane 16). (B) The same blot probed with β-actin cDNA to allow normalization of the amount of RNA. (C) Quantitative measurements of three separate Northern blots using laser densitometry. (D) Northern blot of adult murine tissues probed with pFB8. Each lane contains 40 μg of total RNA from adult lung (lane 1), kidney (lane 2), spleen (lane 3), heart (lane 4), intestine (lane 5), liver (lane 6), brain (lane 7), and human HTB10 (lane 8). The size of the transcript was determined by comparison with a 0.24- to 9.5-kb RNA ladder (GIBCO-BRL). Bar represents the mean ± SD ($n = 3$).

blots were subsequently hybridized to a cDNA probe for β-actin (Fig. 1B). Both the neurofibromin and β-actin signals were quantitated with laser densitometry. In addition, the signal intensity of 18S and 28S rRNA was found to be the same in lanes with RNAs from different developmental ages (data not shown). The relative levels of expression of NF1 transcripts are shown in Fig. 1C. Fetuses of gestational ages E8 through E10 expressed lower levels of NF1 transcripts than older fetuses. At E11, the level of NF1 transcripts was approximately four- to fivefold higher than that at E8 to E10. This level of expression was maintained through E16 (Fig. 1C).

In order to determine whether the high expression of NF1 mRNA transcripts continues into adulthood, we performed Northern blots of adult murine tissues. Figure 1D shows an autoradiograph of a Northern blot of 40 μg of total RNA from adult murine tissues (twice the amount of RNA in Fig. 1A). Although the pFB8 cDNA insert hybridized to a 13.5-kb mRNA transcript in all murine tissues, the amount of NF1 mRNA was significantly different among these tissues. Brain showed the highest relative amounts of NF1 mRNA transcripts (Fig. 1D, lane 7). Moderate levels of NF1 transcripts were detected in the heart, intestine, kidney, and liver. NF1 transcripts were barely detectable in the lung and spleen. A second NF1 transcript at 12.5 kb detected in human HTB10 neuroblastoma cells was not seen in murine tissues (Fig. 1D, lane 8).

Presence of Neurofibromin in Developing Murine Fetuses

To study the distribution of the NF1 gene product, we had previously raised an AB against a synthetic peptide near the C-terminus of the predicted human neurofibromin. This antibody (anti-NF1C) specifically recognizes a 290–350-kDa protein in the human HTB10 cell line, a 250-kDa protein in rodent brain, and detects widespread expression of its antigen in the rat CNS (Huynh *et al.*, 1992). We used this AB to stain NIH-Swiss mouse sections of developmental stages E8 through E16. All reported staining was absorbed out by the addition of 100 μM peptide. To allow a semiquantitative result, all sections were treated at the same time under identical experimental conditions.

Figure 2 shows the low magnification view of paraffin sections stained with the anti-NF1C antibody. In E8 and E9 murine fetuses, only weak staining of embryonic tissues was observed (Fig. 2). In E10 embryos, overall staining increased. In order to correct for different cellular density in specific tissues we compared sections with corresponding sections stained with hematoxylin/eosin (H/E) (not shown). In the primitive nervous system, the first branchial arch (BA), and the primitive heart (atrium and ventricle) staining exceeded the amount expected on grounds of cell density (Fig. 2, E10). On the other hand, the majority of mesenchymal tissue was stained less intensely than that on the corresponding H/E section.

At E11, all embryonic tissues were stained. However,

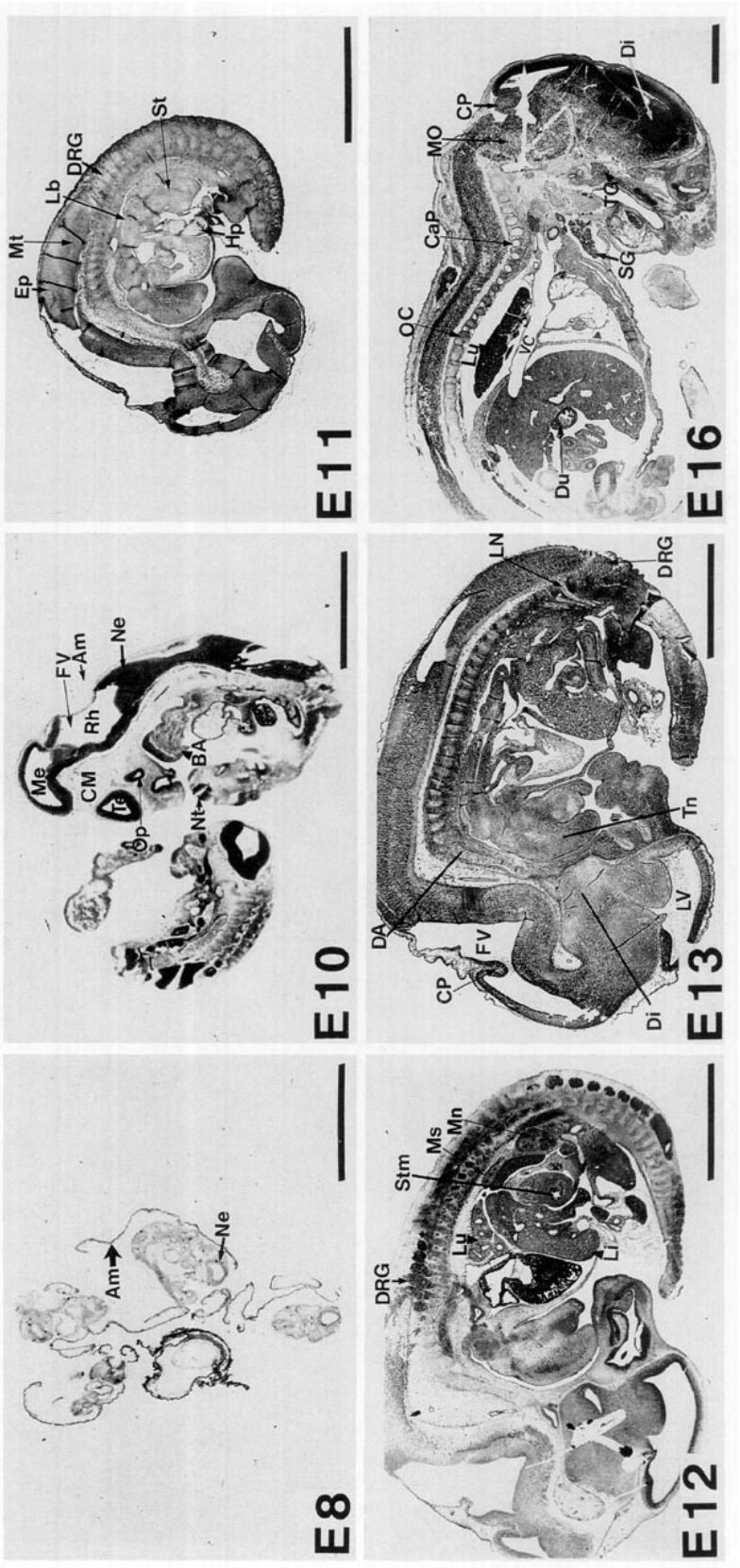

FIG. 2. Detection of neurofibromin in murine fetuses using an antibody NF1C. Sections were representatives of four experiments obtained from two different pregnancies. (Bar, 1.67 mm.) Am, amnion; BA, mandibular part of first branchial arch; CaP, cartilage primordium; CM, cephalic mesenchyme tissue; CP, cerebellar primordium; DA, dorsal aorta; Di, diencephalon; DRG, dorsal root ganglion; Du, duodenum; Ep, ependymal layer; FV, fourth ventricle; Hp, hepatic primordial element; Lb, lung bud; Li, liver; LN, segmental lumbar nerve tract; Lu, lung; LV, lateral ventricle; Me, mesencephalon; Mn, tubules of metanephros; MO, medulla oblongata; Ms, mesonephric tubule; Mt, mantle layer; Ne, neuroepithelium; Nt, neural tube; OC, ossification within cartilage primordium; Op, optic vesicle; Rh, rhombencephalon; SG, submandibular gland; St, stomach; Stm, mucous membrane of stomach; Te, telencephalon; TG, trigeminal (V) ganglion; Tn, muscle mass of tongue; VC, vena cava.

staining in the developing central nervous system was more intense than the staining observed in the heart, lung bud (Lb), dorsal root ganglia (DRG), stomach (St), and hepatic primordium (Fig. 2, E11). At E12 strong IR was observed in the heart, the dorsal root ganglia, the liver, the mesonephric tubules, the metanephros, the epithelial lining of the stomach, and the bronchial tubules (Fig. 2, E12).

From E13 to E16, staining was intense in all embryonic tissues including cells in the dermis and epidermis. However, as shown in the photograph of an E16 embryo, staining was not uniform. In some tissues, such as the lung and the submandibular gland, dark staining was related to high cell density when compared to H/E-stained sections (not shown). In other tissues, however, such as in the nervous system and the buccal epithelium, staining intensity in some regions was unrelated to cell density (see below).

A more detailed analysis of staining in selected tissues is shown in Figs. 3 and 4. At E11, spinal postmitotic neuroepithelia, which will become either neuroblasts or glioblasts, in the mantle layer (Mt) were more intensely stained than the undifferentiated, dividing neuroepithelia of the ependymal layer (Ep) (Fig. 2, E11, and Fig. 3A). Strong staining in the mantle layer was not the result of higher cell density since the corresponding H/E section showed that the ependymal layer in fact has a higher cell density than the mantle layer. The marginal layer (Mr), which consists of processes from neuroepithelia and neuroblasts from the inner layers, was almost devoid of staining (Fig. 3A). Strong staining of cells in the mantle layer and subsequently in spinal grey matter persisted through E16.

Two tissues, the DRGs and the heart, showed rapid changes in neurofibromin content. Although staining in the DRGs was visible at E11, there was a marked increase in staining at E12, followed by a decrease at E13 (Figs. 2 and 3). This lower level of immunoreactivity persisted through E16. Even more marked changes were found in the heart. Whereas at E11 only faint staining was observed in the heart, at E12 the whole organ stained darkly (Figs. 2 and 3C; see also Fig. 6). Already at E13 neurofibromin IR had decreased to lower levels and remained at that level through E16 (compare with the staining of the adjacent liver tissue, Figs. 2 and 3C).

Figure 4 shows differential staining of neurons during formation of the neocortex. Proliferating neurons in the ventricular zone showed barely visible neurofibromin IR. Pronounced IR was seen in neurons in the neopallial cortex (cortical plate) and in some neurons in the intermediate zone (Fig. 4B). As shown in an adjacent H/E-stained section (Fig. 4A) this pattern could not be explained on the basis of cell density.

Differential Expression of Type I and Type II Neurofibromin mRNAs in Developing Fetal Mice

We studied the relative expression of type I and II NF1 mRNAs in mouse fetuses. Since the two transcripts differ only by the insertion of 63 bp in the NF1-GRD region, we chose reverse-transcribed PCR to determine the relative expression of both transcripts in murine fetuses. We synthesized oligonucleotide primers that flank the exon specifying the type II transcript. When type II transcript is present, a PCR product that is 63-bp larger than the type I PCR product is seen. Figure 5A shows the result of a representative experiment. In four separate RNA-PCR experiments, type II neurofibromin transcripts were predominantly expressed in early gestational stages of murine embryos. The level of type II transcript was approximately fourfold higher than type I in gestational ages E8 to E10 (Fig. 5B). At the time when the overall expression of NF1 transcripts increased at E11 (Figs. 1A and 1C), type I transcript became the predominant form (Fig. 5B).

Localization of Type II Neurofibromin in Murine Embryos

To study the distribution of protein products of type II transcripts, we used an antibody (GAP4) against a peptide containing the 21 amino acids encoded by the 63 bp determining the type II NF1 transcript. The AB was affinity-purified as previously described (Huynh *et al.*, 1992). In Western blots of rodent brain tissues, the GAP4-AB detected a band of predicted relative molecular weight of approximately 250 kDa (Fig. 6A, lane 2). In addition, a fainter band of approximately 230 kDa was detected. Both bands were eliminated when the antibody was preabsorbed with 100 μM of the GAP4 peptide (Fig. 6A, lane 1).

Fetal mouse sections adjacent to those used for anti-NF1C antibody immunohistochemistry were stained with the GAP4-AB. In early embryonic stages, the pattern of GAP4-AB immunoreactivity was similar to NF1C-AB staining, although the intensity appeared to be weaker. At E10, neuroepithelia of the primitive CNS, the first BA, and the hepatic primordium (Hp) were intensely labeled (Fig. 6D). Already at E10 cardiac myoblasts lining the primitive heart were labeled with GAP4-AB when compared with corresponding sections stained with NF1C-AB. All staining was absorbed out with the addition of 100 μM of the GAP4 peptide (Fig. 6C), indicating that the GAP4 antibody staining was specific.

In E12 embryos, the overall intensity of staining was much weaker than that observed for the NF1C-AB. How-

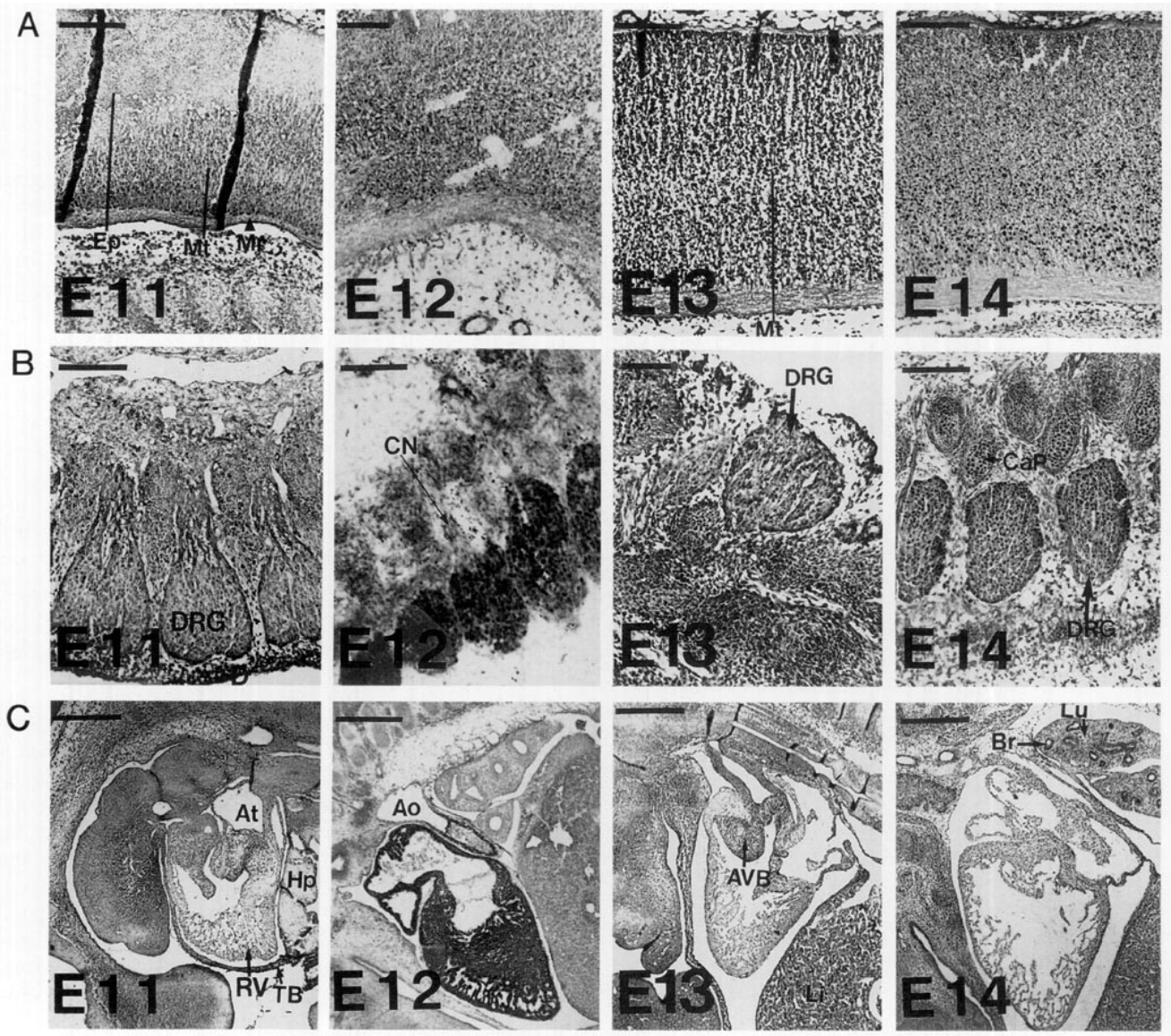

FIG. 3. Relative tissue expression of neurofibromin in developing murine embryos. (A to C) Tissues of murine embryos stained with antibody NF1C. Each panel contains a series of developmental sections of mouse embryo spinal cord (A), dorsal root ganglia (B), and hearts (C). (Bar, 0.420 mm). Ao, arch aorta; At, atrium; AVB, atrioventricular bulbar tissues; Br, bronchial tube; CaP, cartilage primordium; CN, cervical nerve; DRG, dorsal root ganglion; Ep, ependymal layer; Hp, hepatic primordium; Li, liver; Mr, marginal layer; Mt, mantle layer; RV, right ventricle of the heart; TB, thoracic body wall.

ever, several tissues showed strong staining compared to other organs in the same tissue section (Fig. 2, E12, and Fig. 6E). Thus, much type II neurofibromin IR was found in the E12 heart and probably represents the major molecular species in this tissue. As was observed with the NF1C-AB in the nervous system, staining in the mantle layer was stronger than that in the ependymal layer. The only other significant staining in the CNS was seen in neuroepithelial cells near the lateral ventricle, which will form the future neopallial cortex.

In E13 embryos (Fig. 6F) and in later stages (not shown) only weak type II neurofibromin IR was detected. The staining seen lining the ventricular spaces was an edge artifact and could not be absorbed out. The rapid decrease of staining from E12 to E13 in the heart, which had been observed with the NF1C-AB (Fig. 3C) was also observed with the GAP4-AB (Figs. 6E and 6F). Past E13, the only noticeable staining was detected in the liver. Most important, no GAP4-AB staining was observed in neurons of the intermediate zone and corti-

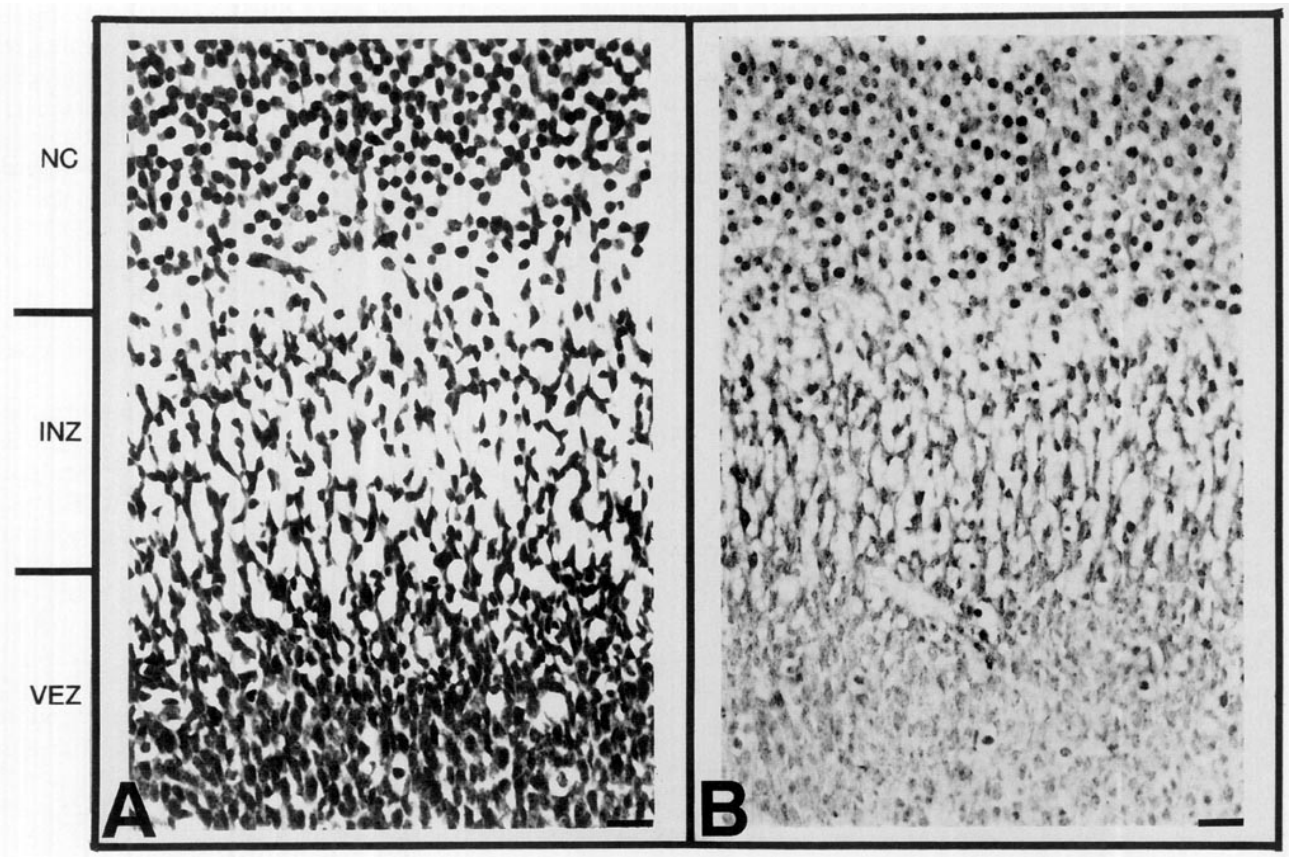

FIG. 4. Expression of neurofibromin is increased in differentiated neurons. (A) The E15 neocortex at the lateral ventricle stained with hematoxylin and eosin. The organization of the neocortex at this stage included the ventricular zone (VEZ) which lines the lateral ventricular space, the intermediate zone (INZ), which contains newly differentiated neurons and nerve tracts, and the neopallial cortex (NC). (B) The corresponding section of the E15 neocortex stained with NF1C antibody. Most differentiated neurons in the cortical plate and the intermediate zone were intensely labeled, while the undifferentiated cells in the ventricular zone were lightly stained. (Bar, 0.042 mm.)

cal plate of the cortex at E15 and E16 (data not shown), whereas strong staining was observed with the NF1C-AB (Fig. 4B).

DISCUSSION

The Expression of Neurofibromin is Developmentally Regulated

Tumor suppressor genes may play an important role in murine development. Biochemical and anatomical evidence for the expression of several tumor suppressor genes has been demonstrated in the embryo (Bernards *et al.*, 1989; Clarke *et al.*, 1992; Donehower *et al.*, 1992; Huang *et al.*, 1990; Jacks *et al.*, 1992; Lee *et al.*, 1992; Rogel *et al.*, 1985). We now provide anatomical evidence showing that the gene product of another tumor suppressor gene, the gene for neurofibromatosis type 1, is abundantly expressed in murine embryos and that it is developmentally regulated.

Neurofibromin transcripts were detected as early as E8 by Northern blotting of total embryonic RNAs (Fig. 1A). Using immunocytochemical methods, neurofibromin-like IR was detected in neuroepithelia of the primitive CNS, the first branchial arch, and the primitive heart in E10 embryos. A majority of mesenchymal tissues, however, were devoid of immunoreactivity from E8 through E10 (Fig. 2, E8, and E10). At E11, a marked change in neurofibromin expression occurred, and neurofibromin transcripts increased approximately four-fold compared to the level at E8 through E10 (Fig. 1). It is not clear why the observed increase in neurofibromin IR in the nervous system was not associated with a clearer increase in NF transcript at E10. Since we chose midsagittal sections to analyze staining in the developing nervous system, and staining was very strong in the E10 nervous system, it is likely that IR was overestimated compared to Northern blots which used whole embryos. It is also possible that neurofibromin is more

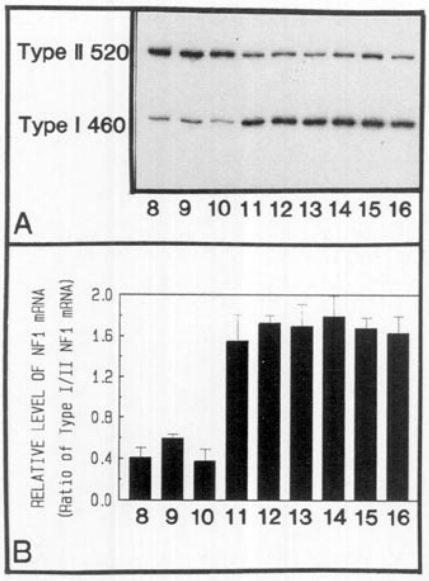

FIG. 5. Relative expression of type I and type II neurofibromin transcripts in developing NIH-Swiss mice. (A) A representative autoradiograph of an RNA-PCR analysis of mouse embryos of different gestational ages. Each lane contains an RNA-PCR sample of 20 ng of murine total RNA from E8 (lane 8), E9 (lane 9), E10 (lane 10), E11 (lane 11), E12 (lane 12), E13 (lane 13), E14 (lane 14), E15 (lane 15), or E16 (lane 16). (B) The densitometric ratios of type I over type II NF1 transcripts determined in four separate experiments. At each stage, four animals in two separated pregnancies were sacrificed to isolate total RNA for these experiments. The bar represents the mean ± SD (n = 4).

stable or more immunogenic in cells at E10 or that the precise timing of embryos used for RNA and anatomical experiments was slightly different. From E11 on, the level of transcript remained relatively constant. Beginning at E11, strong neurofibromin staining was observed outside the nervous system and probably accounted for the increased expression observed in total embryonic mRNAs. However, as discussed below, the increase in neurofibromin expression was not uniform in all tissues.

Developmental Expression of Type I and Type II Neurofibromins and mRNAs

We examined the expression of type I and II neurofibromins which differ by the insertion of 63 bp located in the GRD region of the NF1 gene. We found that the expression of both transcripts was developmentally regulated (Fig. 5). Type II transcripts were predominantly expressed during E8 to E10 gestation, whereas type I transcripts predominated from E11 through E16. Thus, the increase in total neurofibromin transcript at E11 is largely due to an increase in type I neurofibromin.

Since our NF1C-AB was raised against a synthetic peptide common to types I and II neurofibromins we could not determine if some tissues showed a preferential expression of either neurofibromin. To determine if changes in type II neurofibromin expression detected in total embryonic RNA were due to tissue-specific expression, we used the GAP4-AB that recognizes only the 21-aa insert specific for type II neurofibromin. This AB detects a protein of the predicted molecular weight of 250 kDa on Western blots and staining on Western blots and tissue sections could be blocked by preabsorption of the AB with the GAP4 synthetic peptide (Figs. 6A, 6B, and 6C).

As expected, the tissue distribution of staining observed with the GAP4-AB was identical to that observed with the NF1C-AB, i.e., wherever GAP4 staining was observed, staining was also seen with the NF1C-AB (see preceding paragraph). However, some tissues that showed strong staining with the NF1C-AB, for example nonnervous tissues at E13, stained only weakly with the GAP4-AB, indicating that type I neurofibromin was the predominant molecular species.

In E10 embryos, the overall staining pattern for both ABs was similar, indicating that type II neurofibromins constituted a significant portion of total neurofibromin IR. These anatomical observations are consistent with the relative abundance of type II transcripts from E8 to E10. Past E11, staining with the GAP4-AB was barely detectable except for specific tissues (see below).

Expression of Neurofibromin in Relation to Tissue Differentiation

Although neurofibromin was ubiquitously expressed during development, some tissues showed marked and rapid changes in neurofibromin staining. For example, neurofibromin levels in the heart changed significantly between E11 and E13 (Fig. 3). Staining in the E12 heart was mainly due to type II neurofibromin IR (Fig. 6E). Neurofibromin staining increased strongly from E11 to E12 and then rapidly declined at E13. These rapid changes may be related to some of the major developmental changes occurring in the E12 murine heart. These are the formation of the aorticopulmonary septum leading to the formation of the primitive ventricle and the fusion of the septum primum with the atrioventricular cushion tissue resulting in the formation of the atria (Kaufman, 1992). These observations may provide an explanation for the embryonic death at E13 of mice carrying homozygous deletions for the NF1 gene. Preliminary analysis of these embryos indicated that cardiac malformation beginning at E12 resulted in embryonic death 1 to 2 days later (Tyler Jacks, personal communication).

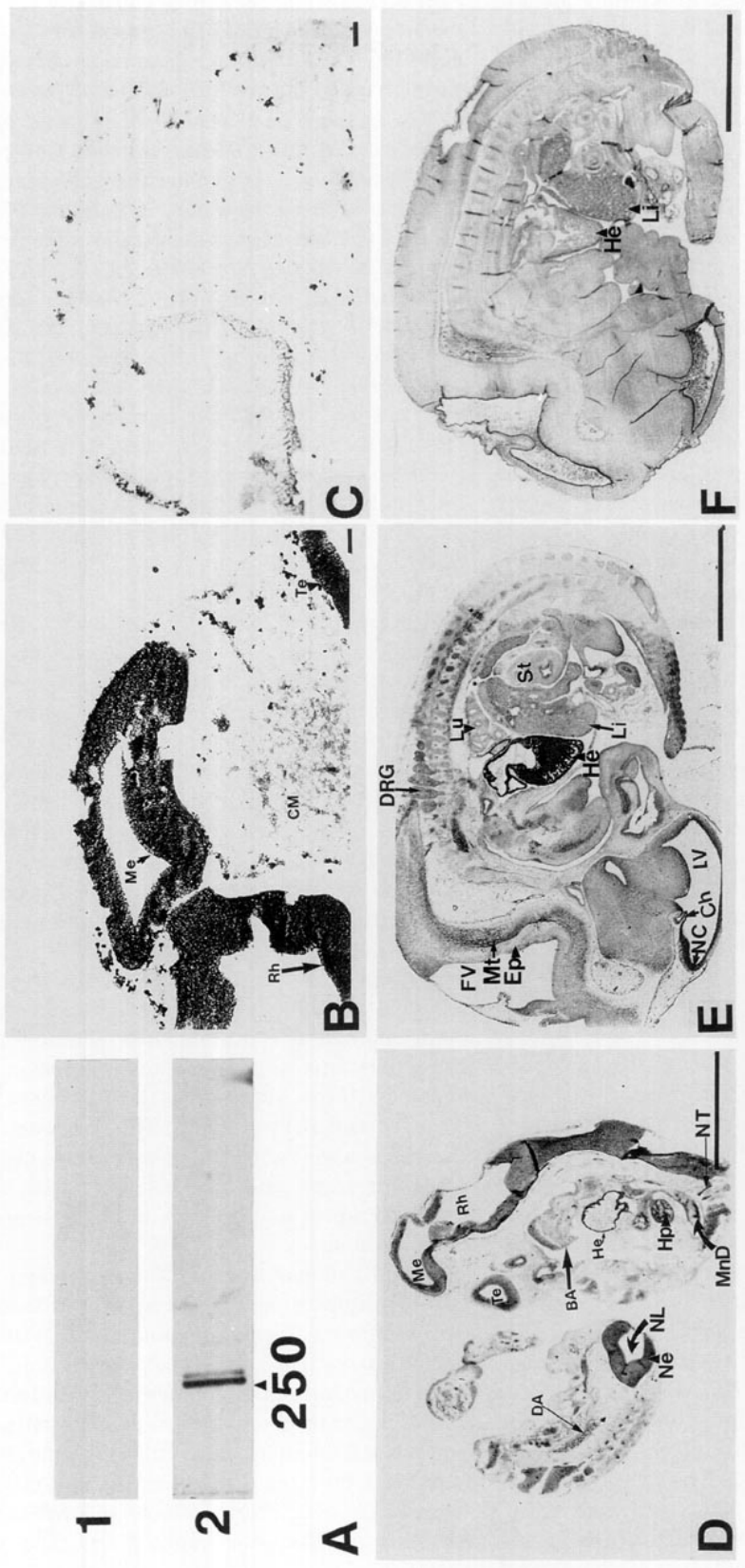

FIG. 6. Expression of type II neurofibromin in murine embryos. Murine embryo sections were stained with an antibody against the 21-aa peptide specific for type II neurofibromin (anti-GAP4). (A) Western blot of adult rodent brain protein extract stained with GAP4 antibody preabsorbed with 100 μM GAP4 peptide (lane 1) and with GAP4 antibody (lane 2). (B) Sagittal section through an E10 head showing staining in neuroepithelia. (C) Adjacent section stained with GAP4 antibody preabsorbed with 100 μM GAP4 peptide. (D) Sagittal E10 mouse section. (E) Sagittal E12 section. (F) Sagittal E13 section which shows weak GAP4 antibody staining. (Bars of B and C, 0.151 mm; bars of D, E, and F, 1.67 mm.) BA, mandibular portion of the first branchial arch; DA, dorsal aorta; Ch, choroid plexus; CM, cephalic mesenchyme tissues; DRG, dorsal root ganglion; Ep, ependymal layer; He, heart; Hp, hepatic primordium; FV, fourth ventricle; Li, liver; LV, lateral ventricle; Lu, lung; Me, mesencephalon; Mt, mantle layer; MnD, mesonephric duct; NC, neopallial cortex; Ne, neuroepithelium; NL, neural lumen; NT, posterior nerve tract; Rh, rhombencephalon; St, stomach; Te, telencephalon.

A similar rapid change occurred in spinal root and trigeminal ganglia. Although neural crest-derived cells expressed high levels of neurofibromin throughout murine development (Figs. 2, 3, and 6), there appeared to be a marked increase in staining at E12, which was followed by a rapid return to pre-E12 levels of staining 24 hr later. This increase of neurofibromin expression may be related to the status of differentiation of ganglion cells. Both spinal and cranial ganglia are highly differentiated by E12 in comparison to other differentiating organs at this stage of development (Kaufman, 1992).

In the nervous system, two examples of rapid changes in neurofibromin levels related to neuronal migration and differentiation were identified. Cells in the ependymal layer of the spinal cord which are still proliferating showed only weak staining with either AB, but cells in the mantle layer were strongly positive for both ABs (Figs. 2, 3A, and 6), indicating the presence of mainly type II neurofibromin. The second example involved type I neurofibromin expression during formation of the neocortex. Neurons in the ventricular zone that are actively undergoing mitoses showed barely visible neurofibromin IR. However, neurons in the intermediate zone and even more pronounced in the cortical plate were darkly stained (Fig. 4). In contrast to mantle layer neurons, no type II neurofibromin IR was detected in cortical neurons. It will be important to examine mice missing neurofibromin for abnormalities in ganglionic, spinal cord, and cortical development. In contrast to cardiac malformations which are not part of the human NF1 phenotype, structural and functional abnormalities in the human CNS are frequently found in patients with NF1 (see references in Introduction).

Function of Neurofibromin in Development

One of the proposed functions of neurofibromin is to accelerate the GTPase activity of ras^{Ha}, ras^{Ki}, and ras^{N} p21 proteins for the conversion of active GTP-bound $p21^{ras}$ to the inactive GDP-bound form (Basu *et al.*, 1992; DeClue *et al.*, 1992; Golubic *et al.*, 1991; Han *et al.*, 1991; Martin *et al.*, 1990; Uchida *et al.*, 1992). An increase of neurofibromin will decrease the level of GTP-bound $p21^{ras}$ (Basu *et al.*, 1992; DeClue *et al.*, 1992) and thus decrease the rate of $p21^{ras}$-dependent cellular proliferation and differentiation (Barbacid, 1987).

Several lines of evidence suggest that ras proteins mediate important developmental functions controlling cell proliferation and differentiation. First, injection of $p21^{ras}$ proteins into *Xenopus* oocytes induced maturation (Birchmeier *et al.*, 1985) and into NIH-3T3 cells stimulated cell growth (Mulcahy *et al.*, 1985; Stacey and Kung, 1984). Second, in *Drosophila* larvae, proliferating tissues expressed high levels of ras2 transcripts (Segal and Shilo, 1986). Tissues which normally lack the *ras* gene developed abnormally in transgenic *D. melanogaster* carrying a mutated *ras 2* gene (Bishop and Corces, 1988). Furthermore, the differentiation of *Drosophila* photoreceptor neurons appeared to be induced by ras proteins through a complex signal transduction pathway regulated by a family of genes called the sevenless receptor tyrosine kinases. Recently, a member of the sevenless family, the son of sevenless, was cloned and its amino acid sequence was similar to that of the CDC25 protein, an activator of ras proteins in *Saccharomyces cerevisiae* (Bonfini *et al.*, 1992). In addition, the amino acid sequence of Gap1, which negatively regulates the sevenless receptor tyrosine kinase pathway is similar to the mammalian ras GTPase activating protein, the yeast IRA1 and IRA2 products, and human neurofibromin (Gaul *et al.*, 1992). In mammalian development, terminal differentiation of PC12 cells was inducible by $p21^{ras}$ (Ha- and Ki-ras) in the presence of extracellular signal-regulated kinases (Qui and Green, 1992; Szeberenyi *et al.*, 1990).

However, despite the presumed role of ras proteins in normal development, there is no evidence that the level of ras proteins is developmentally regulated (Muller *et al.*, 1982, 1983; Slamon and Cline, 1984). The apparent lack of regulation of ras expression during development suggests that other upstream or downstream elements of ras signal transduction pathways are regulated. The regulation of neurofibromin may be one of the ways used to regulate ras-dependent growth signals during development. One would therefore expect that tissues known to express ras proteins abundantly show expression of neurofibromin as well.

Although little is known about the distribution of ras proteins in embryonic tissues, a recent study of mouse embryos from E6.5 to E12 showed abundant expression of ras proteins in neural elements and in the primitive heart, with low expression in other developing tissues (Brewer and Brown, 1992). This pattern of ras expression agrees well with the observed tissue distribution of neurofibromins and is consistent with a function of neurofibromin as a regulator of ras-mediated signal transduction.

In adult mouse and rat, the expression of $p21^{ras}$ was highest in brain, and moderate to low levels were found in nonnervous tissues (Tanaka *et al.*, 1986; Spandidos and Dimitrov, 1985; Leon *et al.*, 1987). Similarly, the *NF1* gene is predominantly expressed in adult rodent CNS, with a lower level of expression in thymus, kidney, liver, and heart (Buchberg *et al.*, 1990; Golubíc *et al.*, 1992). Our Northern blot results confirmed the observation that the adult rodent CNS produces severalfold more neurofibromin mRNA transcripts than other nonneural tissues (Fig. 1D).

The strong evolutionary conservation of the 21-aa insert specifying type II neurofibromin suggests a distinct functional role for this molecular species. Our anatomical studies support this hypothesis by showing that both types are differentially regulated, and that they have a different relative tissue distribution during development (Figs. 2 and 6). Whereas in the heart type II neurofibromin expression is associated with major developmental changes, type I neurofibromin is expressed in neurons once they have reached the cortical plate.

Biochemical studies support this hypothesis as well: First, although both types appear to be able to stimulate the hydrolysis of c-Ha-ras and c-Ki-ras GTP-p21 proteins (Andersen *et al.*, 1993; Uchida *et al.*, 1992; Viskochil and Bollag, personal communication), type II neurofibromin activity is lower. Second, both neurofibromins may interact differently with other regulators. One such regulator is arachidonic acid, which has been shown to be more effective in inhibiting type I than type II NF1-GRD GTPase-activating function (Uchida *et al.*, 1992). Third, neurofibromins may have functions distinct from interacting with ras proteins. For example, neurofibromin was found to be associated with microtubules in rat fibroblasts (Gutmann and Collins, 1992). Since microtubules are thought to play an essential role in cellular proliferation, the interaction of neurofibromin with these skeletal elements may imply a role for neurofibromin in the regulation of cellular division distinct from a ras signal transduction pathway (Seizinger, 1993). Fourth, since the C-terminus of the 21-aa insert is similar to a nuclear signal sequence (Suzuki *et al.*, 1991), type II neurofibromin may also act as a signal for nuclear translocation. Immunohistochemical studies by us and others do not exclude this possibility, because some cells exhibited nuclear as well as perinuclear staining (Golubic *et al.*, 1992; Huynh *et al.*, 1992).

In conclusion, our studies demonstrate that different neurofibromins are expressed during mouse embryogenesis. The activity of $p21^{ras}$ GTPase during murine development may be at least in part regulated through variable expression of neurofibromin. However, neurofibromins may have other functions outside the ras signal transduction pathway, and the functional role of different neurofibromin domains needs to be further determined. In order to test if neurofibromin expression is essential for normal development, it will be necessary to analyze transgenic mice that carry homozygous deletions for the NF1 gene. Our studies may provide the framework to interpret some of the developmental abnormalities observed in these mice. The presence of neurofibromin during mouse development and its developmental regulation are consistent with a major developmental role of neurofibromins, which is suggested by the presence of developmental abnormalities observed in patients with NF1.

We thank Dr. Gengfeng Xu for the pFB8 NF1 cDNA and Drs. David Viskochil and Ray White for the GAP4 antibody. This work was supported by grants from the Steven and Lottie Walker Foundation, the Carmen and Louis Endowment fund, and by National Institutes of Health Grant NS01428.

Note. While this work was in progress the authors became aware of a manuscript (Daston, M. M., and Ratner, N., 1992) which describes total neurofibromin IR in the developing rat nervous system. Their findings are in good agreement with the staining observed with the NF1C-antibody.

REFERENCES

Andersen, L. B., Ballester, R., Marchuk, D. A., Chang, E., Gutmann, D. H., Saulino, A. M., Camonts, J., Wigler, M., and Collins, F. S. (1993). A conserved alternative splice in the von Recklinghausen neurofibromatosis (NF1) gene produces two neurofibromin isoforms, both of which have GTPase-activating protein activity. *Mol. Cell. Biol.* **13**, 478–495.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, R. E., Seidman, J. G., Smith, J. A., and Struhl, K. (1989). "Short Protocols in Molecular Biology." pp. 158–160. Greene Pub. Assoc. and Wiley-Interscience, New York.

Bader, J. L. (1986). Neurofibromatosis and cancer. *Ann. N.Y. Acad. Sci.* **486**, 57–65.

Barbacid, M. (1987). ras Genes. *Annu. Rev. Biochem.* **56**, 779–827.

Basu, T. N., Gutmann, D. H., Fletcher, J. A., Glovers, T. W., Collins, F. S., and Downward, J. (1992). Aberrant regulation of ras proteins in malignant tumor cells from type 1 neurofibromatosis patients. *Nature* **356**, 713–715.

Bernards, R., Schackleford, G. M., Gerber, M. R., Horowitz, J. M., Friend, S. H., Schartl, M., Bogenmann, E., Rapaport, J. M., McGee, T., Dryja, T. P., and Weinberg, R. A. (1989). Structure and expression of the murine retinoblastoma gene and characterization of its encoded protein. *Proc. Natl. Acad. Sci. USA* **86**, 6474–6478.

Birchmeier, J. G., Broek, D., and Wigler, M. (1985). Ras proteins can induce meiosis in *Xenopus* oocytes. *Cell* **43**, 615–621.

Bishop, J. G., and Corces, V. G. (1988). Expression of an activated ras gene causes developmental abnormalities in transgenic *Drosophila melanogaster*. *Genes Dev.* **2**, 567–577.

Bonfini, L., Karlovich, C. A., Dasgupta, C., and Banerjee, U. (1992). The Son of sevenless gene product: A putative activator of ras. *Science* **255**, 603–606.

Brewer, L. M., and Brown, N. A. (1992). Distribution of $p21^{ras}$ in postimplantation rat embryos. *Anat. Rec.* **234**, 443–451.

Buchberg, A. M., Cleveland, S. S., Jenkins, N. A., and Copeland, N. G. (1990). Sequence homology shared by neurofibromatosis type-1 gene and IRA-1 and IRA-2 negative regulators of the RAS cyclic AMP pathway. *Nature* **347**, 291–294.

Cawthon, R. M., Weiss, R., Xu, G., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P., and White, R. (1990). A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* **62**, 191–201.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M. T., van der Valk, M., Hooper, M. L., Berns, A., and te Riele, H. (1992). Requirement for a functional Rb-1 gene in murine development. *Nature* **359**, 328–330.

*Daston, M. M., Scrable, H., Nordlund, M., Sturbaum, A. K., Nissen, L. M., and Ratner, N. (1992). The protein product of the neurofibromatosis type 1 gene is expressed at highest abundance in neurons, schwann cells, and oligodendrocytes. *Neuron* **8,** 415–428.

Daston, M. M., and Ratner, N. (1992). Neurofibromin, a predominantly neuronal GTPase activating protein in the adult, is ubiquitously expressed during development. *Developmental Dynamics* **195,** 216–226.

DeClue, J. E., Cohen, B. D., and Lowy, D. R. (1991). Identification and characterization of the neurofibromatosis type 1 protein product. *Proc. Natl. Acad. Sci. USA* **88,** 9914–9918.

DeClue, J. E., Papageorge, A. G., Fletcher, J. A., Diehi, S. R., Ratner, N., Vass, W. C., and Lowy, D. R. (1992). Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. *Cell* **69,** 265–273.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery Jr., C. A., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356,** 215–221.

Gaul, U., Mardon, G., and Rubin, G. M. (1992). A putative ras GTPase activating protein acts as a negative regulator of signaling by the sevenless receptor tyrosine kinase. *Cell* **68,** 1007–1019.

*Golubic, M., Tanaka, K., Dobrowolski, S., Wood, D., Tasai, M. H., Marshall, M., Tamanoi, F., and Stacey, D. W. (1991). The GTPase stimulatory activities of the neurofibromatosis type 1 and the yeast IRA2 proteins are inhibited by arachidonic acid. *EMBO J.* **10,** 2897–2903.

Golubic, M., Roudebush, M., Dobrowolski, S., Wolfman, A., and Stacey, D. W. (1992). Catalytic properties, tissue and intracellular distribution of neurofibromin. *Oncogene* **7,** 2151–2159.

Gutmann, D. H., and Collins, F. S. (1992). Recent progress toward understanding the molecular biology of von Recklinghausen neurofibromatosis. *Ann. Neurol.* **31,** 555–561.

Gutmann, D. H., Wood, D. L., and Collins, F. S. (1991). Identification of the neurofibromatosis type 1 gene product. *Proc. Natl. Acad. Sci. USA* **88,** 9658–9662.

Hall, A. (1990). ras and GAP—who's controlling whom? *Cell* **61,** 921–923.

Han, J. W., McCormick, F., and Macara, I. G. (1991). Regulation of ras-GAP and the neurofibromatosis-1 gene product by eicosanoids. *Science* **252,** 576–579.

Hattori, S. M., Maekawa, M., and Nakamura, S. (1992). Identification of neurofibromatosis type I gene product as an insoluble GTPase-activating protein toward ras p21. *Oncogene* **7,** 481–485.

Huang, A., Campell, C. E., Bonetta, L., McAndrews-Hill, M. S., Chilton-MacNeill, S., Coppes, M. J., Law, D. J., Feinberg, A. P., Yeger, H., and Williams, B. R. (1990). Tissue, developmental, and tumor-specific expression of divergent transcripts in Wilms tumor. *Science* **250,** 991–993.a.

Hunter, T. (1991). Cooperation between oncogenes. *Cell* **64,** 249–270.

Huynh, D. P., Lin, C. T., and Pulst, S. M. (1992). Expression of neurofibromin, the neurofibromatosis 1 gene product: Studies in human neuroblastoma cells and rat brain. *Neurosci. Lett.* **143,** 233–236.

Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. (1992). Effects of an Rb mutation in the mouse. *Nature* **359,** 295–300.

Kaufman, M. H. (1992). "The Atlas of Mouse Development." Academic Press, San Diego.

Korenberg, J. R., Pulst, S. M., Neve, R. L., and West, R. (1987). The Alzheimer amyloid precursor protein maps to human chromosome 21 bands q21.105–q21.05. *Genomics* **5,** 124–127.

Korf, B. R. (1990). Diagnosis of neurofibromatosis and clinical overview. *In* "Neurofibromatosis: A Handbook for Patients, Families, and Health-Care Professionals" (A. E. Rubenstein and B. R. Korf, Eds.), pp. 15–28. Thieme, New york.

*Kyritsis, A. P., Lee, P. S. Y., Mochizuki, H., Nishi, T., Levin, V. A., and Saya, H. (1992). Differential splicing of the neurofibromatosis type I gene in the rat: Splice variants homologous with the human are expressed in rat cells. *Int. J. Oncol.* **1,** 149–152.

Lee, E. Y. H. P., Chang, C. Y., Hu, N., Wang, Y. C. J., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359,** 288–294.

Leon, J., Guerrero, I., and Pellicer, A. (1987). Differential expression of the ras gene family in mice. *Mol. Cell. Biol.* **7,** 1535–1540.

Marchuk, D. A., Saulino, A. M., Tavakkol, R., Swaroop, M., Wallace, M. R., Andersen, L. B., Mitchell, A. L., Gutmann, D. H., Boguski, M., and Collins, F. S. (1991). cDNA Cloning of the type 1 neurofibromatosis gene: Complete sequence of the NF1 gene product. *Genomics* **11,** 931–940.

Martin, G. A., Viskochil, D., Bollag, G., McCabe, P. C., Crosler, W. J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthon, R. M., Innis, M. A., and McCormick, F. (1990). The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell* **63,** 843–849.

Mulcahy, L. S., Smith, M. R., and Stacey, D. W. (1985). Requirement for ras proto-oncogene functioning during serum-stimulated growth of NIH 3T3 cells. *Nature* **313,** 241–243.

Muller, R., Slamon, D. J., Adamson, E. D., Tremblay, J. M., Muller, D., Cline, M. J., and Verma, I. M. (1983). Transcription of c-onc genes c-ras Ki and c-fms during mouse development. *Mol. Cell. Biol.* **3,** 1062–1069.

Muller, R., Slamon, D. J., Tremblay, J. M., Cline, M. J., and Verma, I. M. (1982). Differential expression of cellular oncogenes during pre- and postnatal development of the mouse. *Nature* **299,** 640–644.

Nishi, T., Lee, P. S. Y., Oka, K., Levin, V. A., Tanse, S., Morino, Y., and Saya, H. (1991). Differential expression of two types of the neurofibromatosis type 1 (NF1) gene transcripts related to neuronal differentiation. *Oncogene* **6,** 1555–1559.

Pulst, S. M. (1991). Prenatal diagnosis of the neurofibromatosis. *In* "Clinics in Perinatology." Saunders, Philadelphia.

Pulst, S. M., Gusman, D., Rothman, B. S., and Mayeri, E. (1986). Coexistence of egg-laying hormone and alpha-bag cell peptide in bag cell neuron of Aplysia indicates that they are a peptidergic multitransmitter system. *Neurosci. Lett.* **70,** 40–45.

Qui, M. S., and Green, S. H. (1992). PC12 cell neuronal differentiation is associated with prolonged $p21^{ras}$ activity and consequent prolonged ERK activity. *Neuron* **9,** 705–717.

Riccardi, V. M. (1981). Von Recklinghausen neurofibromatosis. *N. Engl. J. Med.* **305,** 1617–1627.

Rogel, A., Popliker, M., Webb, C. G., and Oren, M. (1985). p53 cellular tumor antigen: Analysis of mRNA levels in normal adult tissues, embryos, and tumors. *Mol. Cell. Biol.* **5,** 2851–2855.

Segal, D., and Shilo, B. Z. (1986). Tissue localization of *Drosophila melanogaster* ras transcripts during development. *Mol. Cell. Biol.* **6,** 2241–2248.

Seizinger, B. R. (1993). NF1: A prevalent cause of tumorigenesis in human cancers? *Nature Genet.* **3,** 97–99.

Shu, H. H., Mirowitz, S. A., and Wippold II, F. J. (1993). Neurofibromatosis: MR imaging findings involving the head and spine. *Am. J. Roentgenol.* **160,** 159–164.

Slamon, D. J., and Cline, M. J. (1984). Expression of cellular oncogenes during embryonic and fetal development of the mouse. *Proc. Natl. Acad. Sci. USA* **81,** 7141–7145.

Spandidos, D. A., and Dimitrov, T. (1985). High expression levels of ras p21 protein in normal mouse heart tissues. *Biosci. Rep.* **5,** 1035–1039.

Stacey, D. W., and Kung, H. F. (1984). Transformation of NIH 3T3 cells by microinjection of Ha-ras p21 protein. *Nature* **310,** 508–511.

Suzuki, Y., Suzuki, H., Kayama, T., Yoshimoto, T., and Shibahara, S. (1991). Brain tumors predominantly express the neurofibromatosis type 1 gene transcripts containing the 63 base insert in the region coding for GTPase activating protein-related domain. *Biochem. Biophys. Res. Commun.* **181,** 955–961.

Szeberenyi, J., Cai, H., and Cooper, G. M. (1990). Effect of a dominant inhibitory Ha-ras mutation on neuronal differentiation of PC12 cells. *Mol. Cell. Biol.* **10,** 5324–5332.

Tanaka, T., Ida, N., Shimoda, H., Waki, C., Slamon, D. J., and Cline, M. J. (1986). Organ specific expression of ras oncoproteins during growth and development of the rat. *Mol. Cell. Biochem.* **70,** 97–104.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some application. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.

Uchida, T., Matozaki, T., Suzuki, T., Matsuda, K., Wada, K., Nakano, O., Konda, Y., Nishisaki, H., Nagao, M., Sakamoto, C., and Kasuga, M. (1992). Expression of two types of neurofibromatosis type 1 gene transcripts in gastric cancers and comparison. *Biochem. Biophys. Res. Commun.* **187,** 332–339.

Viskochil, D., Buchberg, A. M., Xu, G., Cawthon, R. M., Stevens, J., Wolff, R. K., Culver, M., Carey, J. C., Copeland, N. G., Jenkins, N. A., White, R., and O'Connell, P. (1990). Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell* **62,** 187–192.

Wallace, M. R., Marchuk, D. A., Andersen, L. B., Letcher, R., Odeh, H. M., Saulino, A. M., Fountain, J. W., Brereton, A., Nicholson, J., Mitchell, A. L., Brownstein, B. H., and Collins, F. S. (1990). Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. *Science* **182,** 181–186.

Xu, G., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R., and Tamanoi, F. (1990a). The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of *S. cerevisiae*. *Cell* **63,** 835–841.

Xu, G., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R., and Weiss, R. (1990b). The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell* **62,** 599–608.