Expression of the Neurofibromatosis 1 (*NF1*) Isoforms in Developing and Adult Rat Tissues[1]

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Abstract

The neurofibromatosis 1 (*NF1*) gene encodes a large M_r ~250,000 phosphoprotein, the expression of which in adult tissues is limited to neurons, Schwann cells, oligodendrocytes, adrenal medulla, and leukocytes. The presence of two alternatively spliced exons (23a and 48a) in the *NF1* gene allow for the generation of four possible neurofibromin isoforms. Type 1 neurofibromin contains neither 23a or 48a exon sequences, while type 2 neurofibromin contains only the 23a exon insertion. Previous studies have demonstrated that types 1 and 2 neurofibromin might have different functional properties relative to microtubule association and GTPase-activating protein activity towards p21-ras. To determine the normal pattern of expression of these *NF1* isoforms, the adult and developmental expression of types 1 and 2 *NF1* was examined. Herein, we demonstrate that *NF1* mRNA is expressed at varying levels in adult tissues and is developmentally regulated during embryogenesis. Neurons in the central nervous system express predominantly type 1 *NF1*. Using mouse neocortical cultures enriched for neurons or glial cells, type 1 *NF1* predominance was demonstrated in neurons, while type 2 *NF1* predominated in glial cells. In contrast to central nervous system neurons, neurons expressing the type 2 *NF1* isoform were identified in the developing dorsal root ganglia and spinal cord by *in situ* hybridization using a type 2-specific oligonucleotide probe. The elucidation of the differential expression pattern of these two *NF1* isoforms during development and in adult life provides the foundations for future studies aimed at determining the functions of these neurofibromin isoforms.

Introduction

NF1[3] is a common autosomal dominant disorder in which patients manifest an increased risk of developing both benign and malignant cancers (1). For this reason, the *NF1* gene is thought to function as a tumor suppressor gene. Tumor suppressor genes operate to negatively regulate cell proliferation through a variety of mechanisms ranging from altered interactions with the cytoskeleton to direct regulation of nuclear gene transcription (2). Because these gene products negatively regulate cell proliferation, it is presumed that their expression would be developmentally regulated and, in some cells, increased during periods of active cell differentiation. This observation is true for the *NF1* gene product, neurofibromin, whose expression has been shown to be high during periods of active cell differentiation (3, 4).

The *NF1* gene codes for four neurofibromin isoforms which differ by the use of two alternatively spliced exons (exon 23a and 48a; Ref. 5). These four neurofibromin isoforms include type 1 neurofibromin (lacking both 23a and 48a exon sequences), type 2 neurofibromin (containing only 23a exon sequences), type 3 neurofibromin (containing only 48a exon sequences), and type 4 neurofibromin (containing both 23a and 48a exon sequences). Type 1 neurofibromin has been shown to be an excellent negative regulator of p21-ras and functions *in vitro* and *in vivo* as a GTPase-activating protein (6–8). Its expression has been demonstrated in proliferating neuroblasts and Schwann cells *in vitro* (3, 9). In some cells, this isoform of neurofibromin may associate with cytoplasmic microtubules (10). Type 2 neurofibromin, on the other hand, is a 10-fold less efficient negative regulator of p21-ras, may not associate with cytoplasmic microtubules, and in some cell types, is associated with cell differentiation (4, 11). Types 3 and 4 neurofibromin have been described recently and are restricted to cardiac and skeletal muscle tissues (12, 13).

Previous studies examining the developmental expression of neurofibromin have not distinguished between the various isoforms and therefore presented a one-dimensional view of neurofibromin expression. Since each neurofibromin isoform has a slightly different functional profile, it is important to establish the normal patterns of expression of the individual *NF1* isoforms in adult tissues and during embryogenesis. In this paper, we describe the types 1 and 2 *NF1* isoform expression patterns in adult tissues and during rat embryonic development, with special attention to nervous system tissues. The elucidation of these patterns of isoform expression lays the foundations for future studies aimed at determining the functional consequences of neurofibromin isoform switches during cell differentiation.

Results

***NF1* Isoform Expression in Adult Tissues.** A number of studies have demonstrated that neurofibromin expression predominates in neurons, Schwann cells, oligodendrocytes, adrenal medulla, and WBC during adult life (14, 15). This is in contrast to embryonic development, where neurofibromin expression can be detected in most developing tissues (16, 17; see below). To determine the adult *NF1* isoform expression pattern, selected tissues from adult rats were dissected, and the RNA was prepared for RT-PCR analysis.

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[3] The abbreviations used are: NF1, neurofibromatosis type 1; RT-PCR, reverse transcription-PCR; DRG, dorsal root ganglion; SCG, superior cervical ganglia; CNS, central nervous system.

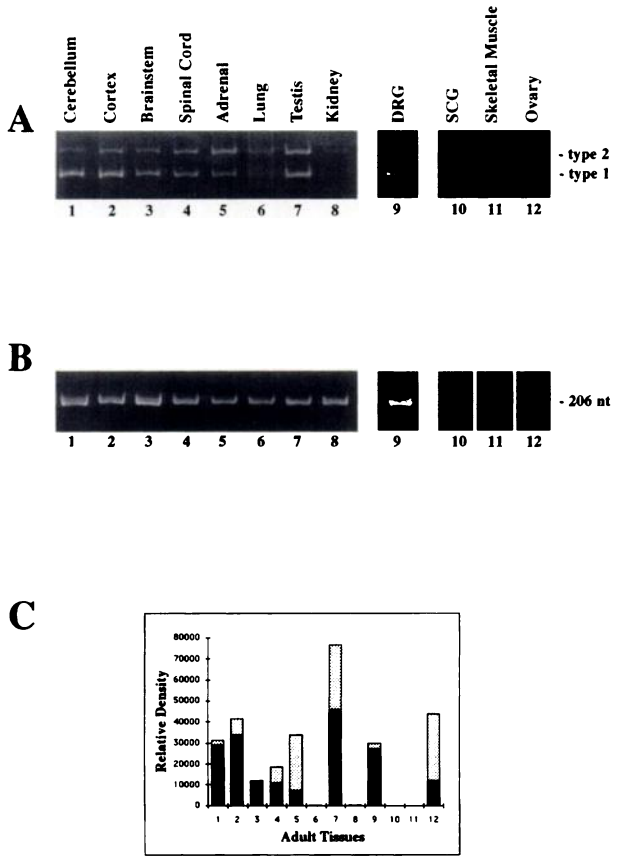

Fig. 1. Expression of *NF1* mRNA isoforms in adult rat tissues. *A*, RNA from selected adult rat tissues were analyzed by RT-PCR using primers that flank the exon 23a insertion to determine the relative abundance and isoform expression pattern of *NF1*. Expression of *NF1* RNA is detected in brain tissues, adrenal gland, DRG, testis, and ovary with nearly undetectable levels in kidney, lung, SCG, skeletal muscle, and heart. The upper band (347 nucleotides) represents type 2 *NF1*, while the lower band (284 nucleotides) represents type 1 *NF1*. *B*, each lane contains an equivalent amount of total RNA as determined by RT-PCR using rat cyclophilin primers. Cyclophilin migrates as a 206-nucleotide RT-PCR product. *C*, graphic representation of the amount of total RNA corrected for the minor variations in cyclophilin and the proportion of each *NF1* isoform as determined by scanning densitometry using NIH-Image 1.53 software. ■, type 1 *NF1*; □, type 2 *NF1*.

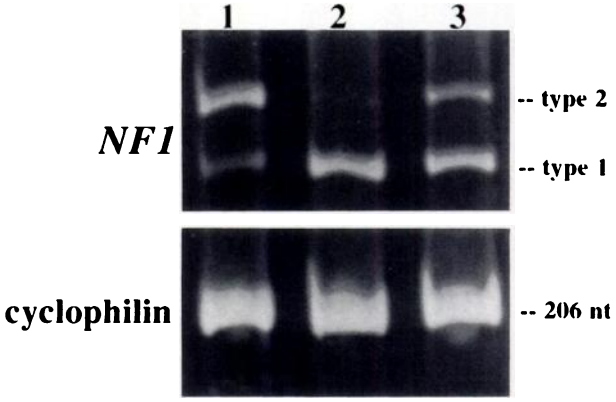

Fig. 2. *NF1* isoform expression in murine cortical cultures. Murine cortical cultures generated to produce pure glial cultures (*Lane 1*), pure neuronal cultures (*Lane 2*), and mixed neuronal-glial cultures (*Lane 3*) were analyzed by RT-PCR. Cyclophilin internal controls demonstrate equal amounts of RNA in each sample. Type 2 *NF1* is predominantly expressed in pure glial cultures, whereas type 1 *NF1* predominates in the pure neuronal cultures. In the mixed cultures, 70–80% of the *NF1* expression is of the type 1 isoform, as determined by scanning densitometry using NIH-Image 1.53 software.

The RT-PCR conditions were chosen to produce semiquantitative information about the relative abundance of total *NF1* mRNA as well as the amount of each *NF1* isoform in each tissue. Under these conditions, there is a linear relationship between the amount of input cDNA and RT-PCR product obtained after 25 cycles of amplification with a 55°C annealing step. In addition, no preferential amplification of types 1 or 2 *NF1* isoform RT-PCR products was observed. This allows for comparison between tissues analyzed at the same time with respect to relative total *NF1* abundance and the proportion of each *NF1* isoform in any given tissue.

A representative experiment is shown in Fig. 1. A number of patterns are readily discernible: (*a*) the *NF1* gene was expressed in many tissues but at varying levels. The use of semiquantitative RT-PCR demonstrates clear differences in levels of total *NF1* expression as well as the ratios of the individual isoforms relative to one and another, in contrast to nonquantitative RT-PCR experiments (11, 18). Little or no expression of the *NF1* gene was detected in lung, kidney, skeletal muscle, SCG, and heart (heart tissue not shown; Ref. 13); and (*b*) different adult tissues exhibited different patterns of *NF1* isoform predominance. The relative proportions of types 1 and 2 *NF1* isoform expression were quantitated by scanning densitometry and illustrated in Fig. 1*C*. The total amount of *NF1* expression was adjusted to reflect the minor differences in cyclophilin expression (Fig. 1*B*; Ref. 19). The highest level of expression observed was in testis. Similar results have been obtained using Northern blot analysis (data not shown). Cerebellum demonstrated 91.6% type 1 predominance, cerebral cortex 80.9%, brainstem 88.2% and DRG 90.5%. Spinal cord and testis exhibited relatively equal amounts of types 1 and 2 *NF1* with 56 and 59.4% type 1 *NF1* isoform predominance, respectively. Adrenal gland and ovary expressed 79.3 and 74.6% type 2 *NF1* isoform predominance, respectively.

Previous work on *NF1* isoform expression in brain tissue has produced conflicting results. Some reports have demonstrated type 1 *NF1* isoform predominance, while others describe type 2 *NF1* isoform predominance (9, 20, 21). Our results demonstrated type 1 *NF1* predominance in adult rat brain tissues but did not clarify which cell in the brain expresses the type 1 or 2 *NF1* isoform. To determine whether neurons and glial cells express different *NF1* isoforms, murine cortical cultures were used (22). Using these cultures, quantitative estimates of the proportion of *NF1* isoform expression in neurons *versus* glial cells can be determined. As can be seen in Fig. 2, there was a type 1 *NF1* isoform predominance in pure neuronal cultures (96.2% type 1 *NF1*) as compared with pure glia, which expressed predominantly type 2 *NF1* (75.5% type 2 *NF1*). Mixed cultures expressed an isoform pattern similar to that seen in brain tissues with 70–80% type 1 predominance. This result argues that most of the *NF1* mRNA expressed in neurons in brain was the type 1 form.

Neurofibromin was shown previously to be expressed in all neurons in the central nervous system with no particular pattern of distribution relative to neurotransmitter content, neuronal pathway, or region of the brain (23). To more

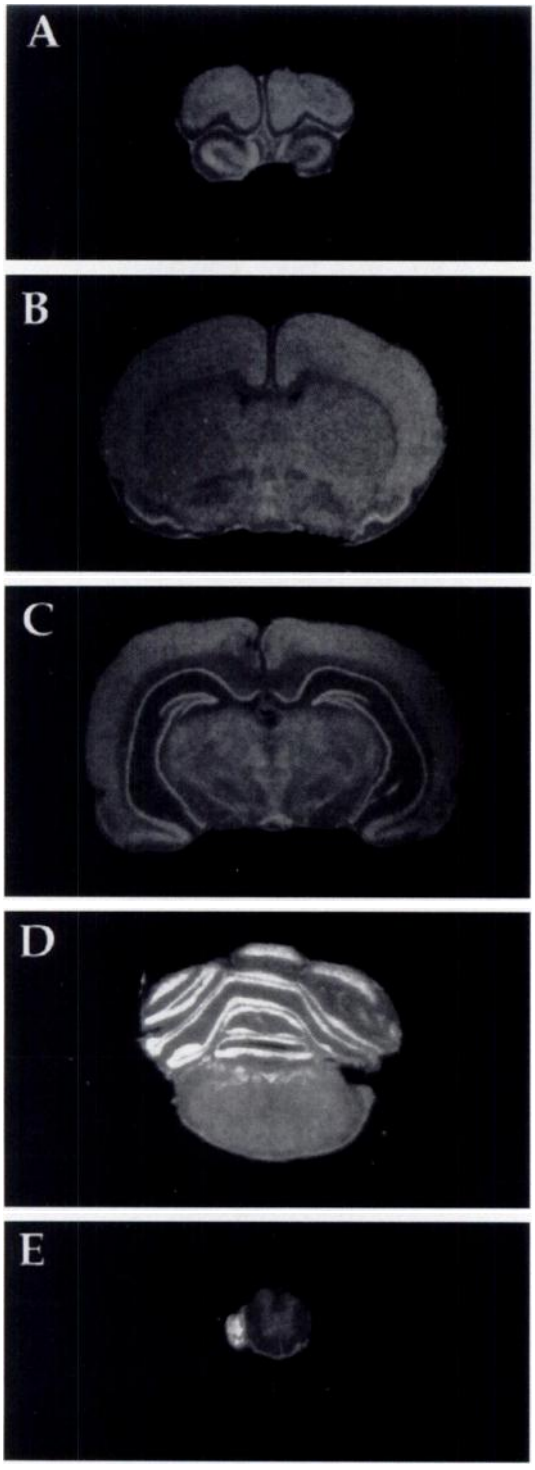

Fig. 3. *NF1* mRNA is expressed by neurons in the adult rat brain. Series of adult rat brain coronal sections were hybridized with the pan-*NF1* antisense riboprobe. Following hybridization, sections were exposed to autoradiographic film, and images were scanned and downloaded onto a MacIntosh Quadra 900 using Adobe Photoshop 2.5.1 software. The *panels* illustrate selected sections through the rat CNS from rostral to caudal and correspond to sections relative to bregma (mm): *A*, +4.7; *B*, −0.26; *C*, −4.80; and *D*, −10.30 (26). In each section, all neurons express *NF1* mRNA with no labeling of white matter tracts. Regions which contain dense cell packing appear brighter. *E*, adult rat spinal cord *NF1* antisense riboprobe hybridization at the level of the upper thoracic cord.

closely examine the pattern of *NF1* mRNA in the central nervous system, sections through adult rat brains were hybridized using a pan-*NF1* riboprobe. In general, all neurons in the adult rat brain appeared to express *NF1* mRNA. Large white matter tracts were unlabeled by the pan-*NF1* riboprobe, including the corpus callosum (Fig. 3, *B* and *C*); anterior commissure (Fig. 3*B*); dorsal, lateral, and ventral funiculi of the spinal cord (Fig. 3*E*); and dorsal roots of the DRG (Fig. 4*H*). We did not quantify levels of mRNA expression since the levels of *NF1* expression varied among different populations of neurons. This may be due to the density of cells within a certain population, giving the appearance that select neurons express *NF1* mRNA at higher levels than others. Cell populations that expressed *NF1* mRNA at the highest levels include mitral cells of the olfactory bulb, neurons in the anterior olfactory nucleus (Fig. 5*A*), anterior hippocampal rudiment (Fig. 5*B*), piriform cortex (Fig. 5*C*), hippocampus (Fig. 5, *D* and *E*), interpeduncular nucleus, substantia nigra, cerebellar Purkinje cells (Fig. 5*G*), frontal cortical neurons (Fig. 5*H*), ventral horn motor neurons (Fig. 4, *A-D*), and DRG cells (Fig. 4, *E-H*). This pattern of expression is consistent with results reported by others using immunohistochemistry and Western immunoblotting (15, 23).

***NF1* Isoform Expression during Embryogenesis.** Previous studies have demonstrated low levels of *NF1* gene expression prior to embryonic day 11 (E11) by Northern and Western immunoblot analysis (4, 16, 17). After this time, there is a gradual increase in *NF1* gene expression in many tissues, including heart, lung, skeletal muscle, and kidney, where little *NF1* gene expression is detected in the adult. Whereas *NF1* RNA can be detected by RT-PCR at embryonic stages prior to E11, there is a gradual and striking increase in the levels of *NF1* mRNA expression after E11, with maximal expression peaking around E16. Western immunoblotting using total protein extracted from whole rat and mouse embryos likewise demonstrated the expression of neurofibromin by E12 in the mouse (16, 17). Expression of *NF1* mRNA can also be detected at low levels throughout rat embryos at E11 by *in situ* hybridization (data not shown). Similar to the immunoblotting results, there was an increase in expression by *in situ* hybridization in all tissues until E16, when maximal *NF1* gene expression was observed. In selected tissues, there was an increase in *NF1* mRNA expression after E16 concomitant with a decrease in expression in other tissues (see below).

To determine the relative expression in representative tissues during late embryogenesis and in early postnatal life, individual organs were removed from rats at different developmental stages, and the RNA was analyzed by semiquantitative RT-PCR. As can be see in Fig. 6, there was a clear developmental pattern of *NF1* isoform expression in all tissues examined. In tissues rich in neurons, like cerebral cortex, brainstem, and cerebellum, there was early and persistent *NF1* expression with a type 1 isoform predominance. The spinal cord demonstrated an initial type 1 predominance, followed by a shift to an equal amount of types 1 and 2 *NF1* mRNA by the end of the first week of postnatal life. This shift in isoform predominance likely reflects the high level of expression of type 2 *NF1* in ventral horn cells (see below).

Other tissues such as DRG and SCG exhibited different patterns of isoform expression. Little expression of *NF1* was seen in SGC during the first 2 weeks of postnatal life with no expression in the adult. DRG, on the other hand, demon-

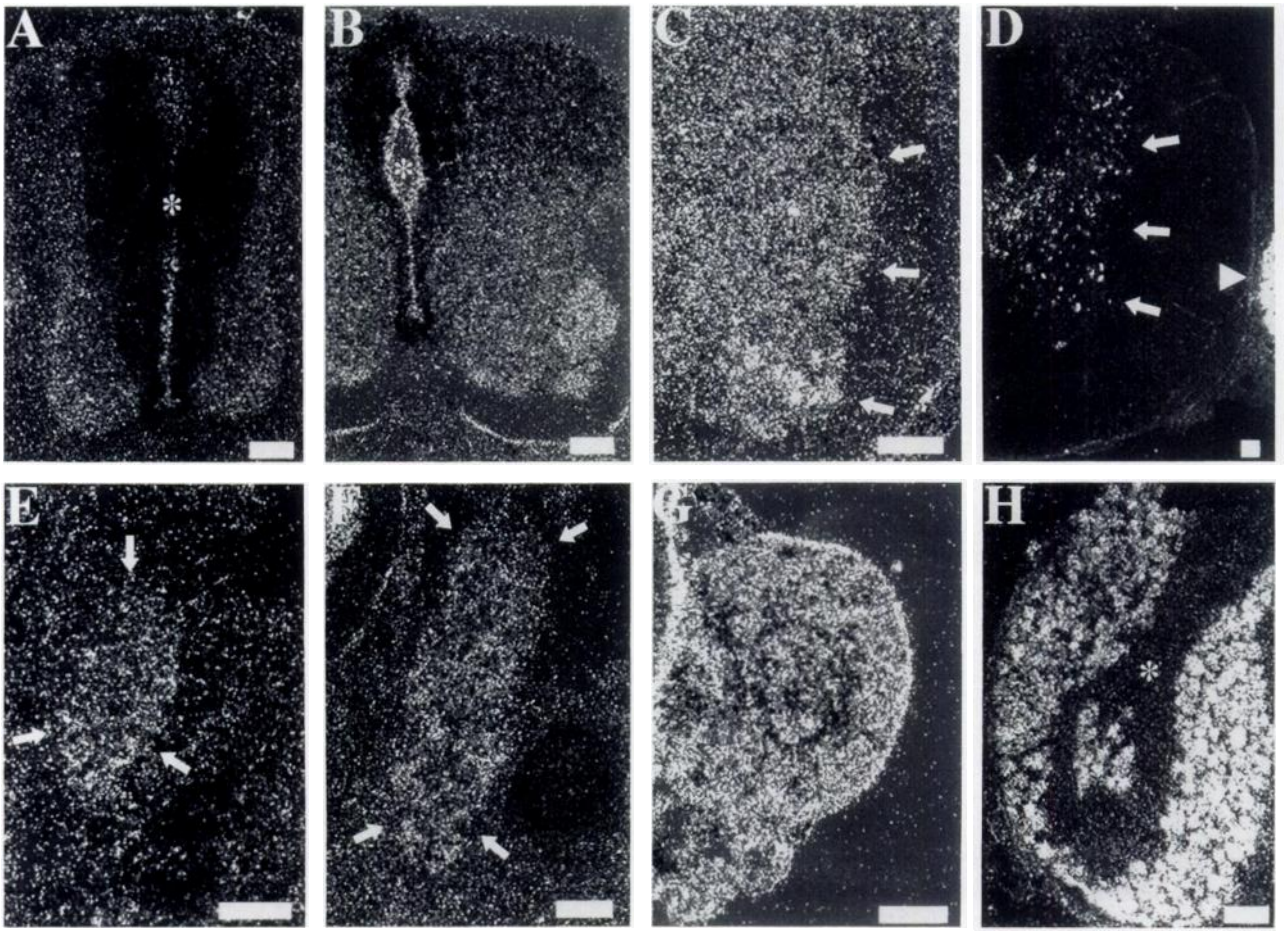

Fig. 4. *NF1* expression in the developing spinal cord and dorsal root ganglion. Dark-field photomicrographs of embryonic and adult transverse sections that were hybridized with the pan-*NF1* antisense riboprobe. *NF1* mRNA is expressed in the developing spinal cord at embryonic days E13 (*A*), E15 (*B*), postnatal day 1 (*C*), and adult (*D*). *C* and *D*, hemisections of the spinal cord with the gray matter indicated by the *arrows*. *, the central canal of the spinal cord; *arrowhead*, the DRG (*Panel D*). *NF1* mRNA is expressed by all primary sensory neurons in the DRG at embryonic days E13 (*E*), E15 (*F*), postnatal day 1 (*G*), and adult (*H*). *E* and *F*, *arrows*, delineation of the borders of the DRG. *H*, *, the dorsal root fibers. Sense and RNase controls failed to demonstrate any specific hybridization signals. *Scale bars*, 100 μm in each panel.

strates a gradual increase in *NF1* expression, beginning at the end of the first week and continuing into adulthood. In the DRG, as in other populations of neurons, there is a type 1 *NF1* isoform predominance, with some cells expressing the type 2 *NF1* isoform. This increase in DRG *NF1* expression parallels an increase in *NF1* mRNA expression in the developing DRG by *in situ* hybridization using a pan-*NF1* riboprobe (Fig. 4, *E-H*). The increase in type 2 *NF1* isoform expression in the DRG was confirmed by *in situ* hybridization (see below). No particular neuronal subpopulations lacking *NF1* mRNA expression could be identified within the DRG.

Examination of the *NF1* isoform expression pattern in developing spinal cord demonstrated an increase in the expression of type 2 *NF1* over time (Fig. 4, *A-D*; Fig. 6). We originally interpreted this to reflect an increase in the number of myelin-producing cells during spinal cord maturation, since both Schwann cells and oligodendrocytes express predominantly type 2 *NF1* (Fig. 2). However, *in situ* hybridization using the type 2 *NF1* oligonucleotide probe clearly demonstrated high levels of type 2 *NF1* expression in motor neurons of the ventral horn (Fig. 7*A*). This is in contrast to the *NF1* expression pattern in the developing spinal cord using the pan-*NF1* riboprobe in which all neurons in the spinal cord expressed *NF1* mRNA (Fig. 4, *A-D*). Similarly, most, but not all, DRG neurons expressed type 2 *NF1* by *in situ* hybridization (Fig. 7*B*). This is in contrast to neurons in the brain, which expressed little type 2 *NF1* by RT-PCR and lacked demonstrable type 2 *NF1* expression by *in situ* hybridization (Figs. 1, 6, and 7*D*). High levels of type 2 *NF1* expression were detected in the E15 rat lung (Figs. 6 and 7*C*).

Expression of *NF1* mRNA was considerable during development and during the first 7–14 days of postnatal life in some tissues lacking adult *NF1* expression. This included heart (13), skeletal muscle, kidney, and lung. In adrenal gland, there was an increase in *NF1* expression after E16 with a type 2 isoform predominance. Expression in the ovary began to rise after postnatal day 1 and was abundantly expressed in adult ovaries. *NF1* expression was also seen in the developing testis, with an increase in type 1 *NF1* observed during the first 2 weeks of life, culminating in equal amounts of each *NF1* isoform in adult testicular tissue.

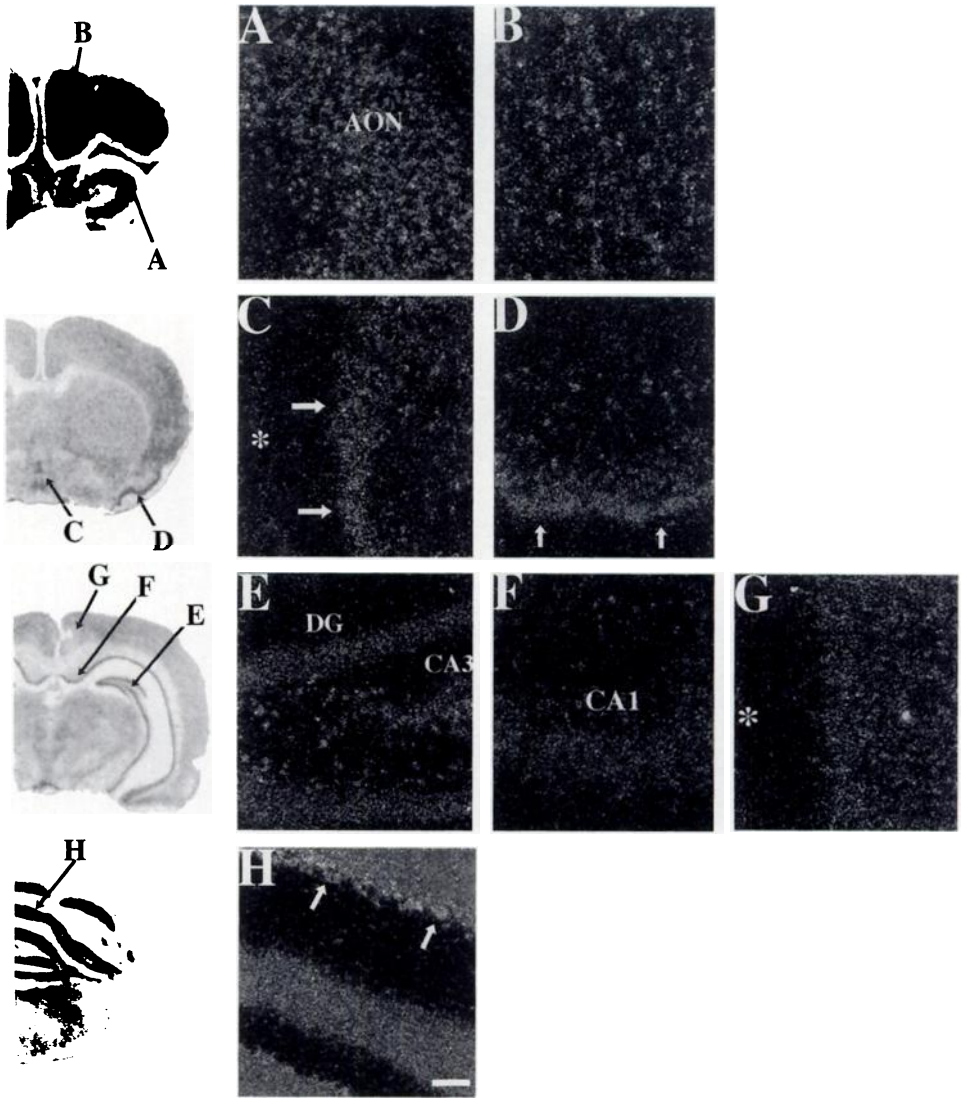

Fig. 5. *NF1* mRNA is expressed by neurons in the adult rat brain. High-power dark-field photomicrographs of selected brain regions from adult rat brain coronal sections hybridized with the pan-*NF1* antisense riboprobe. The area shown in the high power bright field images derive from the appropriately marked regions of the brain denoted with the same letter. Brain slices depicted along the *left side* of the figure represent bright field images of *NF1* RNA expression using the pan-*NF1* antisense riboprobe. Neuronal populations which heavily express *NF1* mRNA include: *A*, anterior olfactory nucleus (*AON*); *B*, frontal cortex; *C*, anterior hippocampal rudiment (*arrows*); *D*, piriform cortex (*arrows*); *E* and *F*, hippocampus; *G*, cingulate cortex; and *H*, cerebellum (*arrows* indicate Purkinje cells); *, *Panels C* and *G*: midline of the section. DG, dentate gyrus; *CA1* and *CA3*, regions of hippocampus. *Scale bars*, 100 μm for each panel.

Discussion

***NF1* Isoform Expression Patterns in Adult Tissues.** Using semiquantitative RT-PCR, we demonstrated that *NF1* mRNA is expressed mainly in neurons of the cerebral cortex, brainstem, cerebellum, spinal cord, DRG, adrenal gland, and gonadal tissues in adults. High levels of expression in adult rat and human testis are also detected by Northern blot analysis at levels 1.5- to 2-fold higher than brain tissues.[4] The role of neurofibromin in developing testis and ovary has not been explored. Little or no expression of *NF1* mRNA is detected in adult kidney, lung, skeletal muscle, and SCG, as described in other studies at the protein level (14, 15).

In adult tissues, type 1 *NF1* predominates in tissues rich in neurons, such as the cerebral cortex, cerebellum, brainstem, and DRG. Using the murine neocortical culture system, cultures of pure neurons, pure glial cells, or mixed cultures containing both neurons and glia were examined to determine their *NF1* isoform expression pattern. Pure cortical neuronal cultures which contain >95% neurons and relatively few glial cells express predominantly type 1 *NF1*, whereas pure glial cultures express predominantly type 2 *NF1*. Many neural crest-derived tissues, such as Schwann cells and adrenal medullary cells, predominantly express the type 2 *NF1* isoform. Previous work demonstrated that Schwann cells differentiating *in vitro* in response to elevations in intracellular cAMP levels change their isoform expression pattern from type 1 to type 2 *NF1* predominance (3). It is possible that this isoform switch is related to an unidentified unique function of neurofibromin in these neural crest-derived cells. Mixed neuronal-glial cultures express the same proportion of type 1 to type 2 *NF1* as cerebral cortex (roughly 70–80% type 1 *NF1* isoform predominance), suggesting that the expression of type 2 *NF1* in glial cells and type 1 *NF1* in neurons contributes to the overall pattern of *NF1* isoform expression in these tissues.

The function of neurofibromin in neurons has not yet been elucidated. Given previous reports that type 1 neu-

[4] D. H. Gutmann, unpublished data.

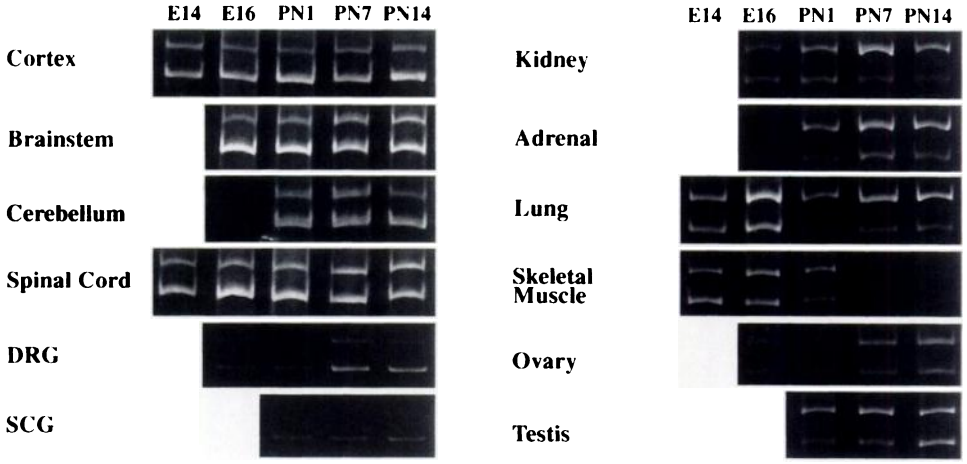

Fig. 6. *NF1* isoform expression during rat embryonic development. RNA from selected rat tissues at various stages of embryogenesis and postnatal life were analyzed by RT-PCR using primers that flank the exon 23a insertion to determine the relative abundance and isoform expression pattern of *NF1*. Each lane contains an equivalent amount of total RNA as determined using cyclophilin primers. Expression of *NF1* RNA is detected in brain tissues (cerebral cortex and brainstem) by E14-E16, whereas expression in the cerebellum increases after E16. Spinal cord expression undergoes a switch in *NF1* isoform expression from type 1 predominance to an equal predominance of *NF1* isoforms during the first week of postnatal life. Expression in the DRG, adrenal gland, testis, and ovary increase after birth, with testis exhibiting an increase in type 1 *NF1* expression after postnatal day 7. Skeletal muscle, kidney, SCG, and lung express *NF1* mRNA during the first week of postnatal life but lack appreciable expression in their adult tissue counterparts. The upper band (347 nucleotides) represents type 2 *NF1*, while the lower band (284 nucleotides) represents type 1 *NF1*.

rofibromin associates with cytoplasmic microtubules (10), it is possible that neurofibromin participates in microtubule-mediated signaling pathways in neurons. However, studies by Nordlund *et al.* (23) have failed to demonstrate colocalization of neurofibromin with neuronal microtubules by electron microscopy (23). Other possible role(s) for neurofibromin neurons may relate to neurotransmitter-mediated signal transduction. Studies in our laboratory have demonstrated no enrichment of neurofibromin in rat brain postsynaptic density or synaptosome fractions, suggesting that neurofibromin might be localized to a different compartment within the neuron. Future studies aimed at determining the function of neurofibromin in neurons will need to address the relationship between neurofibromin, p21-ras down-regulation, and neuronal differentiation.

***NF1* Isoform Expression during Embryonic Development.** During embryonic development, most tissues show no change in the *NF1* isoform expression pattern. In cerebral cortex, brainstem, and cerebellum, type 1 *NF1* predominance is observed throughout embryogenesis. There is no evidence for an isoform switch, either by *in situ* hybridization in E11–E17 rat embryos (data not shown) or by RT-PCR analysis. These results suggest that, in most neurons in the central nervous system, a switch in isoform expression does not contribute to the maturation or differentiation of these cells. However, in the DRG and spinal cord motor neurons, there is an increase in type 2 *NF1* expression over development as documented by RT-PCR and *in situ* hybridization. Neurons with high levels of type 2 *NF1* can be identified in both of these tissues and may represent a unique group of neurons with different properties than their central nervous system counterparts that express predominantly type 1 *NF1*. Neurons in the DRG and spinal cord project to targets outside the CNS, in contrast to the neurons in the brain whose axons are contained entirely within the CNS. It is possible that the expression of the type 2 *NF1* isoform in these peripheral nervous system neurons, but not their CNS counterparts who express mostly type 1 *NF1*, may distinguish these two types of neurons. Future work will focus on these type 2 *NF1*-expressing neurons to determine whether differences in isoform expression reflect different functions for neurofibromin in these populations of neurons.

In the developing kidney, lung, muscle, and heart, there is abundant expression of *NF1* by RT-PCR through the first 1 to 2 weeks of life but little or no expression in adult tissues. This is consistent with protein data demonstrating little neurofibromin expression in adult kidney, muscle, heart, or lung. The expression of *NF1* in the developing ovary and testis suggests that *NF1* expression is also associated with the maturation of these tissues. Moreover, there is a clear change in the *NF1* isoform expression pattern in the developing testis, with type 2 *NF1* predominance during the first 1 to 2 weeks of postnatal life, followed by an increase in type 1 *NF1* isoform expression by postnatal day 14 and in adults. Future studies will be required to determine the role of neurofibromin and its isoforms in gonadal maturation, spermatogenesis, and the estrus cycle.

The expression of multiple isoforms of neurofibromin suggests that this important negative growth regulator may have different functions in different cells as well as different roles during different times of embryonic development. With the establishment of the normal adult and embryonic developmental patterns of *NF1* isoform expression, future studies based on these patterns can be designed to determine the possible functions of neurofibromin during development and cell differentiation.

Materials and Methods

Tissues. Adult and embryonic mouse and rat tissues were obtained from freshly euthanized timed-pregnant and adult animals according to the guidelines established by the

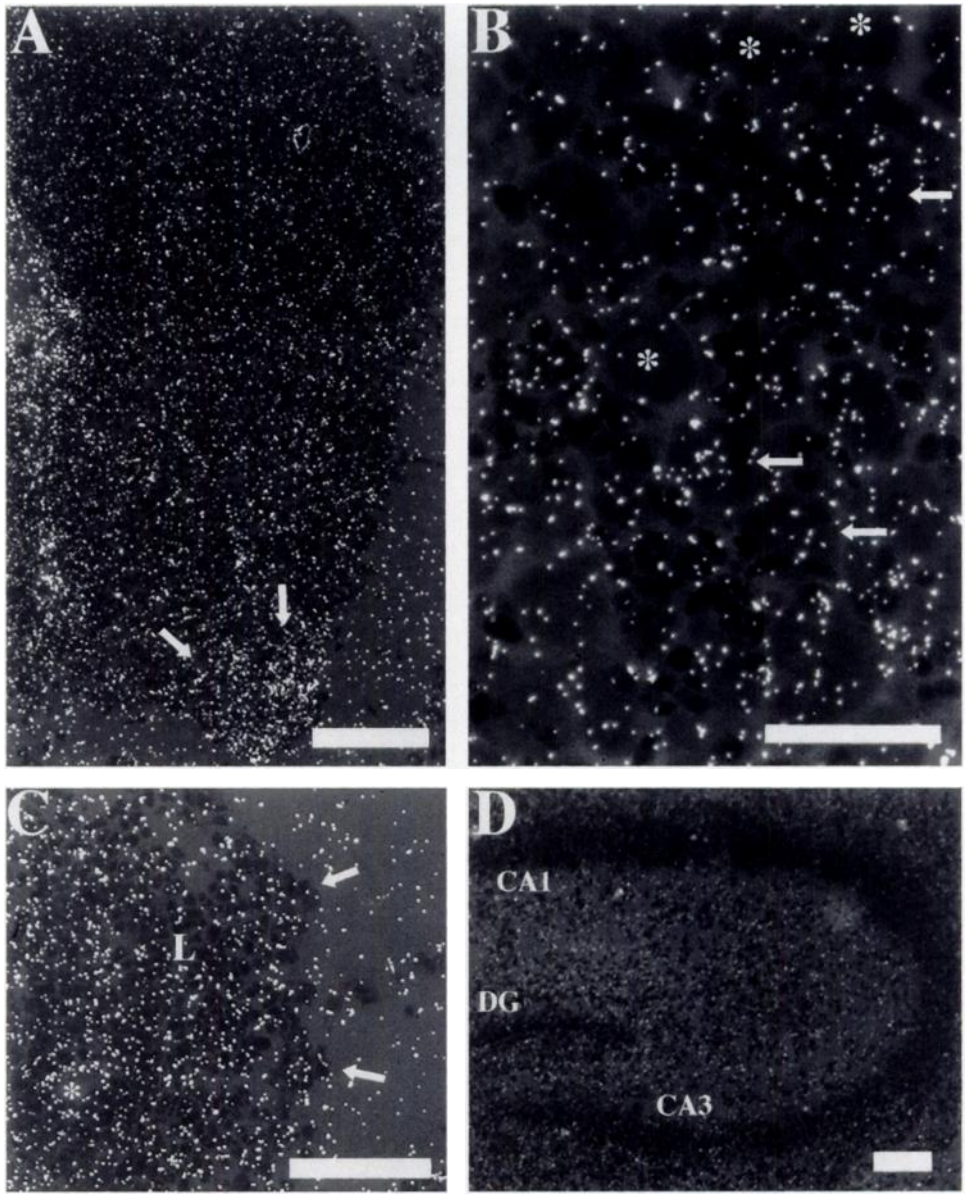

Fig. 7. NF1 type 2 mRNA expression in selected rat tissues during embryonic development. Rat tissue sections were hybridized with a ^{35}S-labeled oligonucleotide probe specific to the *NF1* type 2 exon 23a inserted sequence. *A*, type 2 *NF1* is expressed throughout the developing spinal cord (E15) at low levels. High levels of type 2 isoform expression are detected in motor neurons of the ventral motor pool (*arrows*). *B*, type 2 *NF1* is expressed in some (*arrows*) but not all (*) sensory neurons of the postnatal day DRG. *C*, type 2 *NF1* is expressed in the developing lung at embryonic day E15, as demonstrated by RT-PCR in Fig. 6. *Arrow*, the dorsal surface of lung tissue (*L*); *, a bronchiole. *D*, type 2 *NF1* is not expressed in developing rat brain neurons. This section represents a transverse section at the level of the hippocampus from a postnatal day 1 rat. *DG*, dentate gyrus; *CA1* and *CA3*, regions of hippocampus. This expression pattern is in contrast to the intense labeling observed with the pan-*NF1* riboprobe (Fig. 5*D*) and substantiates the RT-PCR results, demonstrating a type 1 *NF1* isoform predominance in CNS neurons. *Scale bars:* 100 μm in *A*, *C*, and *D*; 50 μm in *B*. Sense and RNase controls failed to demonstrate any specific hybridization signals.

Department of Comparative Medicine at Washington University.

Murine Neocortical Cultures. Murine cortical cultures were established and maintained as described previously (22). Briefly, mixed cortical cultures containing both neurons and glia were prepared from E16 mouse cerebral cortex and cultured in media stock containing modified Eagle's medium supplemented with 2 mM glutamine, 25 mM glucose, and 10% fetal bovine serum. Glial cell cultures from mouse cortex were prepared from 1- to 3-day old pups and cultured on Primaria plates in media stock containing 10% fetal bovine serum, 10% horse serum, and 10 ng/ml epidermal growth factor. Pure neuronal cultures were grown on surfaces coated with poly-D-lysine and laminin in media containing glial culture conditioned medium and 3 μM 1-β-D-arabinofuranosylcytosine. The neurons in these cultures mature, in terms of neurite outgrowth, during the first week *in vitro*. The RNA was extracted from these pure neuronal cultures, mixed glia-neuronal cultures, and pure glial cultures as described below.

Riboprobe Production. A cDNA containing nucleotides 7847 to 8457 of the *NF1* gene was cloned into pBlueScriptII.KS(–). This cDNA recognizes all isoforms of *NF1* RNA. To synthesize antisense and sense riboprobes, this plasmid was linearized with the appropriate restriction endonucleases (*Sal*I for sense and *Xba*I for antisense) and transcribed *in vitro* in the presence of 50 μCi [^{35}S]UTP (Amersham) using either T7 (sense) or T3 (antisense) RNA polymerases. Sense (5'-GCAACTTGCCACTCCCTACTGAATAAAGCTACAGTAAAA GAAAAAAAG-3') and antisense (5'-CTTTTTTTCTTTTACTG-TAGCTTTATTCAGTAGG GAGTGGCAAGTTGC-3') type 2 oligonucleotide probes were synthesized and labeled using terminal deoxyribonucleotide transferase in the presence of [^{35}S]ATP (Amersham).

***In Situ* Hybridization.** The *in situ* hybridization procedures used in our laboratory follow the method described

elsewhere (24). Briefly, frozen sections of rat embryonic tissues were hybridized overnight at 55–60°C with either antisense or sense riboprobes (1×10^6 cpm/slide) diluted in 60 µl of hybridization mixture containing 50% deionized formamide, 10% dextran sulfate, 1X Denhardt's solution, 4X SSC (sodium chloride-sodium citrate solution), 10 mM DTT, 1 mg/ml yeast tRNA, and 1 mg/ml denatured salmon sperm DNA. The following day, the slides were rinsed twice in 4X SSC for 30 min at 37°C, treated for 30 min at 45°C with RNase A (40 µg/ml), and then rinsed at 37°C in 2X SSC (twice for 20 min), 0.5X SSC (twice for 20 min), and 0.1X SSC (30 min). For oligonucleotide probes, frozen sections were hybridized overnight at 37°C with either sense or antisense probes at 1×10^6 cpm/slide. The next day, slides were washed four times in 1X SSC at 55°C, followed by another wash in 1X SSC at room temperature. 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 NBT-2 liquid emulsion and stored in desiccated light-tight boxes at 4°C for 10–30 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 probes. Sections were incubated with individual sense strand ^{35}S-labeled probes or were pretreated with RNase A (Boehringer Mannheim; 20 µg/ml for 30 min at 37°C), followed by hybridization with individual antisense probes. In each case, control hybridizations resulted in complete loss of the hybridization signal. Sections were viewed using dark-field microscopy, and all images were acquired on a Nikon Microphot FXA microscope using a Kodak DCS 200 digital camera and Adobe Photoshop 2.5.1 software for MacIntosh.

RT-PCR. Total cellular RNA was extracted from freshly frozen adult and embryonic rat tissues by the RNasol B technique as per the manufacturer's instructions (Tel-Test, Inc). Each tissue represents organs from at least three rats and has been repeated at least three times for each adult tissue. RNA was precipitated and resuspended in diethyl pyrocarbonate-treated water; the concentration was determined by absorbance at 260 nm and was stored at –80°C until use. Three µg of total tissue RNA was reverse-transcribed as described previously (3, 25). The cDNA products were then amplified by 25 cycles of PCR at an annealing temperature of 55°C. The rat *NF1* primers spanning the exon 23a insertion have been described previously and amplify *NF1* sequences from mice, rats, and human reverse-transcribed RNA (3, 25). Rat cyclophilin primers (5′-ATGGTCAACCCCACCGTGTT-3′ and 5′-CGTTGTAAGTCACCACCCT-3′) amplify a product of 206 nucleotides. All RT-PCR samples contained roughly equivalent amounts of cyclophilin product as an indicator of total RNA quantity per tissue sample (19). The conditions for RT-PCR were chosen based on experiments demonstrating a linear relationship between input reverse-transcribed RNA and the PCR product generated. PCR products were separated on 8% polyacrylamide gels and visualized by ethidium bromide staining.

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