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Differential Tissue-Specific Expression of Neurofibromin Isoform mRNAs in Rat[1]

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We have cloned the full-length cDNA encoding the rat homolog of type I neurofibromin isoform, a protein product of a gene linked to neurofibromatosis type 1. Rat type I neurofibromin isoform is composed of 2,820 amino acid residues and shares about 98.5% amino acid identity with the human counterpart. By S1 nuclease mapping analysis of the alternatively spliced neurofibromin mRNAs in adult rat tissues, we showed that type I isoform mRNA was predominantly expressed in the brain, pituitary, and testis, while type II mRNA, carrying a 63-nucleotide insertion in the region coding for the domain related to GTPase-activating protein, was predominantly expressed in most other tissues, such as heart, kidney, and ovary. Furthermore, type II mRNA is predominant in the testis at age 1 week, while type I mRNA becomes predominant at 3 weeks and is subsequently expressed at higher levels, as seen in the adult testis. In contrast, type I mRNA is the predominant form in the brain throughout the postnatal development. Thus, the relative expression levels of type I and type II mRNAs may be specific to the tissues and to the developmental stage of certain tissues.

Key words: alternative splicing, brain, neurofibromatosis type 1, ras GTPase-activating protein, testis.

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder, and is characterized by *café-au-lait* skin spots, multiple neurofibromas, and higher incidence of malignancy (*1*). A gene linked to NF1 encodes a protein of 2,818 amino acids (*2*), termed neurofibromin. A central portion of neurofibromin is a domain structurally related to GTPase-activating protein (GAP), and was shown to stimulate intrinsic GTPase activity of ras protein (*3-7*). NF1 primarily affects cells of neural crest origin and is considered to be a condition caused by abnormal differentiation of neural crest cells, but neurofibromin is ubiquitously expressed in various human tissues (*8*). Recently, we showed by transient cotransfection assays that overexpression of a putative full-length cDNA encoding rat neurofibromin increased the expression of a reporter gene linked to the human tyrosinase gene promoter (*9*). Tyrosinase [EC 1.14.18.1] is a rate-limiting enzyme in melanin biosynthesis (*10*) and is specifically expressed in melanin-producing cells, such as melanocytes of neural crest origin. We also suggested that the GAP-related domain (GRD) may be mainly responsible for the stimulatory effects of neurofibromin on the tyrosinase promoter activity (*9*). It is therefore conceivable that a neurofibromin-dependent signal may affect transcription of certain genes.

The human neurofibromin gene gives rise to multiple transcripts generated by alternative splicing, coding for neurofibromin and its isoforms. These include amino-terminal isoform mRNAs (*11, 12*), type I mRNA coding for authentic neurofibromin, type II mRNA with a 63-base insertion at the region coding for the GRD (*13-16*), and a 3′ alternatively spliced transcript with a 54-base insertion at the region for the carboxy terminus (*17*). Recently, RNA editing on human neurofibromin mRNA was reported (*18*), indicating that a small fraction of neurofibromin mRNA undergoes base-modification RNA editing, creating an in-frame translational termination codon near the amino-terminal portion of the GRD. However, only limited information is available concerning the rat neurofibromin isoform transcripts, because the nucleotide sequence of a full-length rat neurofibromin cDNA has not been reported.

Among the neurofibromin isoforms so far identified, type II neurofibromin isoform, containing a 21 amino acid insertion at the GRD, has been best characterized. It was reported with a short region of GRD that both type I and type II GRDs have GAP activity (*19*), although the catalytic activity of type II GRD is lower than that of type I GRD (*15*). Thus, much interest has focused on the functions of full-length neurofibromin isoforms (type I and type II),

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Abbreviations: NF1, neurofibromatosis type 1; GAP, GTPase-activating protein; GRD, GAP-related domain; PCR, polymerase chain reaction.

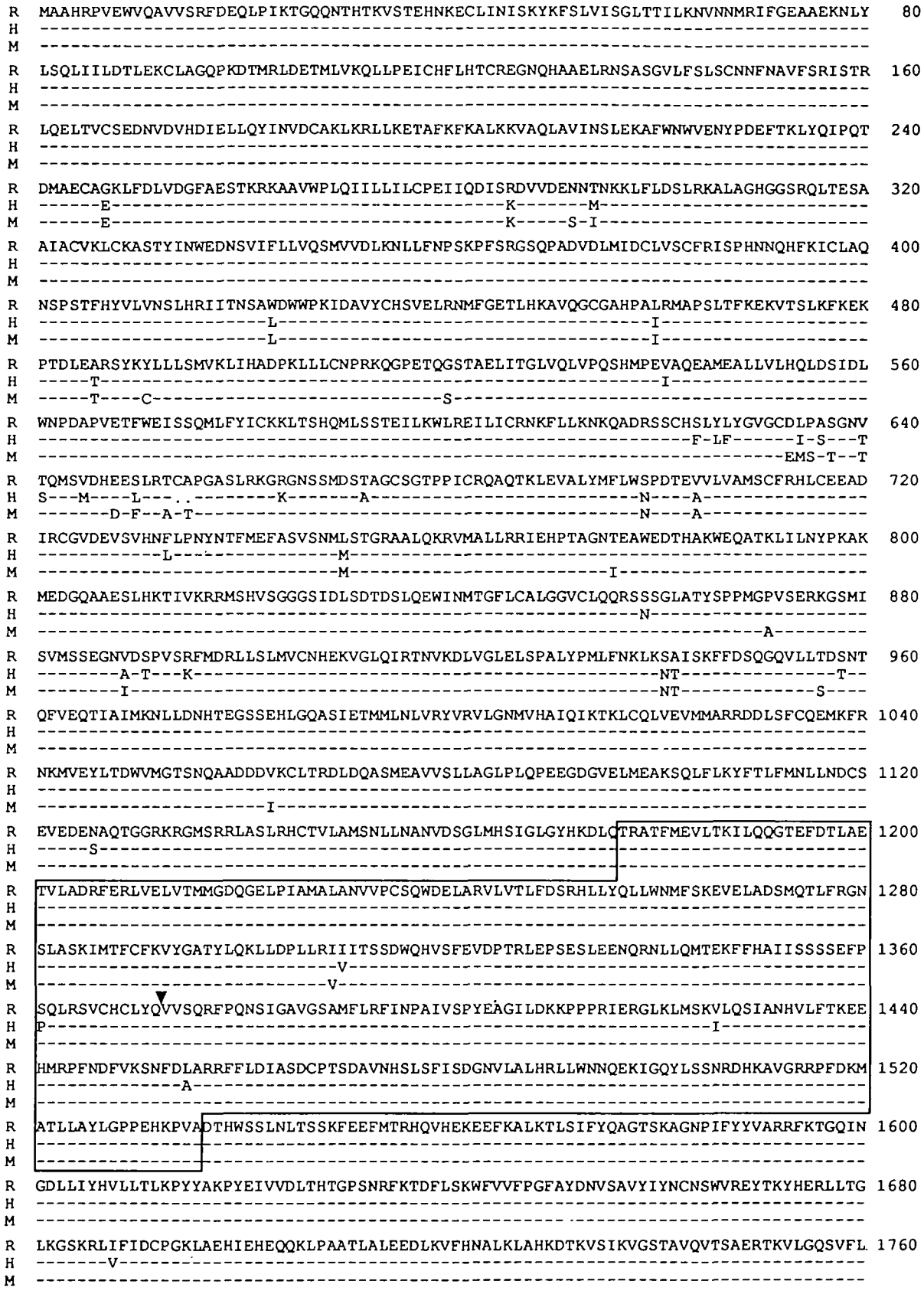

Fig. 1 (continued on next page)

although these have not been fully investigated. On the other hand, the relative expression levels of authentic neurofibromin (type I) and type II isoform mRNAs were reported to change in developing mouse embryo (*20*), in developing chicken brain (*21*), and during neural differentiation of cultured human cells (*13*). In rat, however, the expression levels of type I and type II mRNAs have not been determined.

In this study, we determined the nucleotide sequence of the full-length rat type I neurofibromin cDNA and deduced its complete amino acid sequence. In an effort to assess the physiological role of neurofibromin, we determined the expression levels of type I and type II neurofibromin mRNAs in various rat tissues, including developing brain and testis.

MATERIALS AND METHODS

Molecular Cloning of Rat Neurofibromin cDNA—Rat brain cDNA libraries, constructed in λgt11 or λZAP II vector, were screened with ^{32}P-labeled cDNA fragments derived from human neurofibromin cDNAs as described previously (*9*). Three overlapping cDNA clones were obtained: pRNF2-1 containing the 4.2-kb insert encoding the amino-terminal region of neurofibromin, pRNF-GRD carrying the 2.6-kb insert coding for the GRD, and pRNF3-1 containing the 3-kb insert encoding the carboxy-terminal region (*9*). DNA sequences were determined in both directions using the Taq Dye Primer Cycle Sequencing Kit (ABI) and a 373A automated DNA sequencer (ABI). The nucleotide sequence data reported in this paper have been deposited in the GSDB/DDBJ/EMBL/NCBI DNA databases with the accession number D45201. The expression

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R  NDIYYASEIEEICLVDENQFTLTIANQGTPLTFMHQECEAIVQSIIHIRTRWELSQPDSIPQHTKIRPKDVPGTLLNIAL 1840
H  ------------------------------------------------------------------------------- 
M  ------------------------------------------------------------------------------- 
R  LNLGSSDPSLRSAAYNLLCALTCTFNLKIEGQLLETSGLCIPANNTLFIVSISKTLAANEPHLTLEFLEECISGFSKSSI 1920
H  ------------------------------------------------------------------------------- 
M  ------------------------------------------------------------------------------- 
R  ELKHLCLEYMTPWLSNLVRFCKHNDDAKRQRVTAILDKLITMTINEKQMYPSIQAKIWGSLGQITDLLDVVLDSFIKTSA 2000
H  ------------------------------------------------------------------------------- 
M  ------------------------------------------------------------------------------- 
R  TGGLGSIKAEVMADTAVALASGNVKLVSSKVIGRMCKIIDKTCLSPTPTLEQHLMWDDIAILARYMLMLSFNNSLDVAAH 2080
H  ------------------------------------------------------------------------------- 
M  ------------------------------------------------------------------------------- 
R  LPYLFHVVTFLVATGPLSLRASTHGLVINIIHSLCTCSQLHFSEETKQVLRLSLTEFSLPKFYLLFGISKVKSAAVIAFR 2160
H  ------------------------------------------------------------------------------- 
M  --------------------------L------------------------------------------------------ 
R  SSYRDRSFSPGSYERETFALTSLETVTEALLEIMEACMRDIPTCKWLDQWTELAQRFAFQYNPSLQPRALVVFGCISKRV 2240
H  ------------------------------------------------------------------------------- 
M  ------------------------------------------------------------------------------- 
R  SHGQIKQIIRILSKALESCLKGPDTYNSQVLIEATVIALTKLQPLLNKDSPLHKALFWVAVAVLQLDEVNLYSAGTALLE 2320
H  ------------------------------------------------------------------------------- 
M  ------------------------------S------------------------------------------------ 
R  QNLHTLDSLRIFNDKSPEEVFMAIRNPLEWHCKQMDHFVGLNFNSNFNFALVGHLLKGYRHPSPAIVARTVRILHTLLTL 2400
H  ------------------------------------------------------------------------------- 
M  ------------------------------------------------------------------------------- 
R  VNKHRNCDKFEVNTQSVAYLAALLTVSEEVRSRCSLKHRKSLLLTDISMENVPMDTYPIHHGDPSSRTLKETQPWSSPRG 2480
H  ----------------------------------------------------------Y------------K- 
M  ----------------------------------------------------------Y------------K- 
R  SEGYLAATYPAVGQTSPRARKSMSLDMGQPSQANTKKLLGTRKSFDHLISDTKAPKRQEMESGITTPPKMRRVAETDYEM 2560
H  ----------T-------------------------------------------------------------- 
M  ------------------------------------------------------------------------------- 
R  ETQRISSSQQHPHLRKVSVSESNVLLDEEVLTDPKIQALLLTVLATLVKYTTDEFDQRILYEYLAEASVVFPKVFPLVHN 2640
H  ------------------------------------------------------------------------V--- 
M  -----P-------------------------------------------------------------------V--- 
R  LLDSKINTLLSLCQDPNLLNPIHGIVQSVVYHEESPPQYQTSYLQSFGFNGLWRFAGPFSKQTQIPDYAELIVKFLDALI 2720
H  ------------------------------------------------------------------------------- 
M  ------------------------------------------------------------------------------- 
R  DTYLPGIDEETSEESLLTPTSPYPPALQSQLSITANLNLSNSMTSLATSQHSPGIDKENVELSPTTGHCNSGRTRHGSAS 2800
H  ------------------------------------------------------------------------------- 
M  ----------------------------------------------------L----------A-------------- 
R  QVQKQRSAGSFKRNSIKKIV*                                                           2820
H  --------------------*
M  --------------------*
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Fig. 1. **Deduced amino acid sequence of rat neurofibromin and comparison with human and mouse neurofibromin.** Predicted amino acid sequences of rat, human (*2, 24*), and mouse (*25*) type I neurofibromin are shown at top (R), middle (H), and bottom (M), respectively. The deduced amino acids are numbered beginning with the initiating methionine, and identical amino acids are indicated with small horizontal bars. Note that two successive amino acids are not present in the human sequence, and these are shown with dots. The GRD of 360 amino acids is enclosed. The arrowhead indicates the position of the insertion of 21 amino acids present in the type II neurofibromin isoform. Asterisks indicate the termination codons.

plasmid pRc/RNF, containing a rat full-length type I neurofibromin cDNA under the cytomegalovirus promoter, was constructed as described previously (*9*).

RNA Extraction and S1 Nuclease Mapping—Sprague Dawley adult rats weighing 150–200 g and newborn rats (1 to 8 weeks old) were killed under the ether anesthesia and various tissues were removed. The tissues were stored at −80°C until RNA extraction. Total RNA was extracted from tissues by guanidium thiocyanate-cesium chloride method. S1 nuclease mapping analysis was performed as described previously (*22*). The S1 probe was prepared from rat brain cDNA by polymerase chain reaction (PCR). The sense primer was 5′-CGGAACCTCCTTCAGATGAC-3′ (positions 4009 to 4028) and the anti-sense primer was 5′-ATCCCTGCTTCATACGGTGA-3′ (complementary to positions 4195 to 4214). The primer set used was designed based on the human neurofibromin cDNA sequence (*2*) and was subsequently found to be identical to the rat sequence, except for the C residue at position 4009, at which the rat sequence contains an A residue (GSDB/DDBJ/EMBL/NCBI DNA databases with the accession number D45201). The nucleotide residues were numbered according to the published sequence of human neurofibromin (*2*) and the 63-bp insert of type II mRNA is located between the positions 4114 and 4115. The nucleotide sequence of this 63-bp insert was identical to the reported rat sequence (*16*). The amplified cDNA fragment was inserted into the *Eco*RV site at position 697 of pBluescript II KS vector (Stratagene, CA, USA), and its nucleotide sequence was confirmed by the dideoxy chain-termination method (*23*) using a BcaBEST sequencing kit (Takara, Kyoto). During this procedure, the *Eco*RV site was recovered at the 3′-end of the insert DNA. A subclone thus obtained contains the cDNA insert representing type II neurofibromin mRNA. The *Pvu*II/*Eco*RV fragment of 549 bp was isolated from this clone and was end-labeled at the *Eco*RV site with [γ-^{32}P]ATP (7,000 Ci/mmol, ICN). The *Pvu*II site is located at position 977 in the vector. Total RNA (10 μg) was hybridized with the S1 probe for 3 h at 42°C and digested with 20 units of S1 nuclease (Boehringer-Mannheim) for 30 min at 37°C. The conditions of S1 nuclease digestion were assessed by the presence of the labeled *Eco*RV blunt-end of the reannealed S1 probe. The protected fragments were analyzed on a 5% polyacrylamide gel containing 7 M urea. Radioactive signals were analyzed by exposing the dried gel to X-ray films (X-AR5, Kodak). The intensity of hybridization signals of type I and type II mRNAs was also determined by photostimulated luminescence with a Bioimage Analyzer (BAS 2000, Fuji Film, Tokyo).

RESULTS

Sequence analysis of the three overlapping rat neurofibromin cDNAs revealed a long reading-frame coding for 2,820 amino acid residues with a molecular mass of 317 kDa (Fig. 1), which initiates with the ATG codon (nucleotide residues 1-3) and terminates with the TGA codon (nucleotide residues 8461-8463). The length of the cloned cDNA insert is 9,132 bp. Because the deduced GRD contains no insertion of the 21 amino acid residues, the cloned cDNA represents type I neurofibromin mRNA (*13, 14*). The nucleotide sequence shows about 91% overall identity and 96% identity in the protein-coding region to the published sequences of human (*2, 24*) and mouse type I neurofibromin cDNAs (*25*), respectively. No typical RNA editing sites (*18*) were found on the entire region of rat neurofibromin mRNA. Rat type I neurofibromin shares 98.5% amino acid identity with human type I neurofibromin and is longer than human type I neurofibromin by two amino acid residues (Fig. 1). Mouse type I neurofibromin also codes for 2,820 amino acid residues (*25*), sharing 98.7% identity with its rat counterpart.

The nucleotide sequence coding for the rat GRD, amplified by PCR (*16*), is identical to the sequence determined in this study, except for the two bases. Our cDNA, pRc/RNF, contains T and C residues at positions 4358 and 4458,

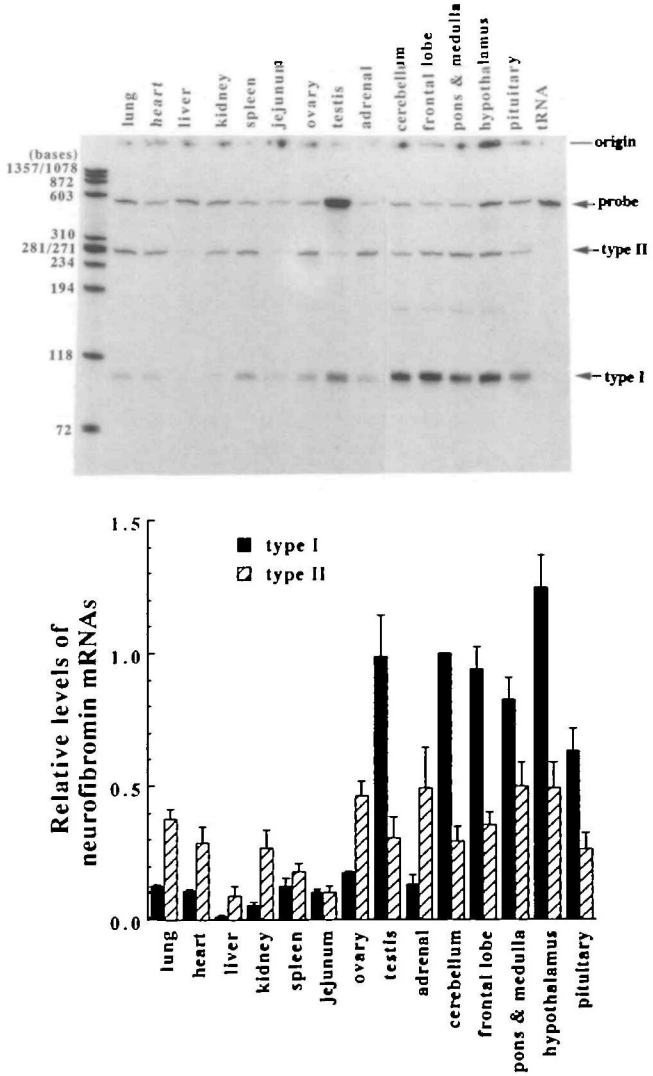

Fig. 2. **Differential expression of mRNAs coding for type I and type II neurofibromin isoforms in adult rat tissues.** Relative expression levels of type I and type II neurofibromin mRNAs were determined in adult rat tissues by S1 nuclease mapping analysis. The fragments of about 100 and 269 bases represent type I and type II mRNAs, respectively. The 549-bp fragments represent the undigested S1 probe. A representative result is shown at top. The data are also shown at bottom as the ratio of the intensity of the band corresponding to type I or type II mRNA in each tissue to that of type I mRNA in the cerebellum. The mean ± SEM of the relative levels of neurofibromin mRNAs in four independent experiments are shown.

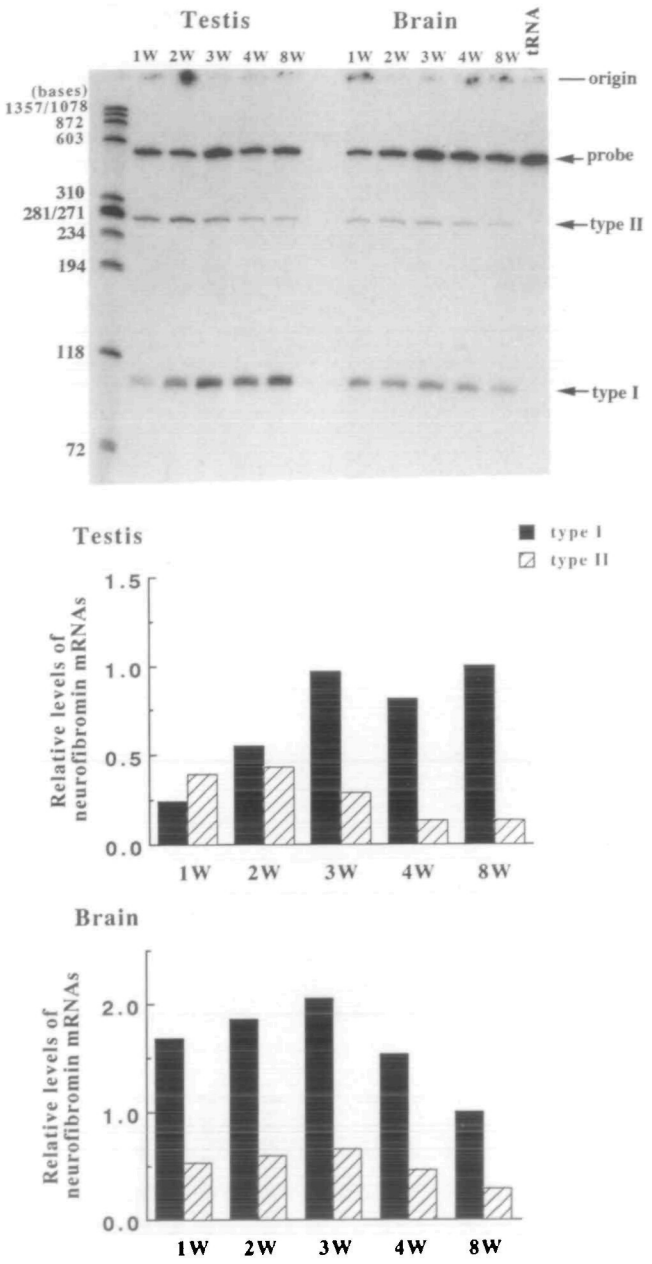

Fig. 3. **Postnatal developmental changes in the relative expression levels of type I and type II neurofibromin isoform mRNAs.** Shown at top is a representative result of the S1 nuclease mapping analysis of neurofibromin isoform mRNAs in the testis and whole brain obtained from newborn rats of the indicated age (from 1 to 8 weeks old). Relative expression levels of type I and type II neurofibromin mRNAs are also shown at bottom as the ratio of the intensity of the band corresponding to type I or type II mRNA in the testis or brain to that of type I mRNA in the respective 8-week tissue.

whereas the published sequence contains C and T residues, respectively. The base change at position 4458 is a silent change. The base change at position 4358 leads to an amino acid substitution at position 1453 (Fig. 1), in which a serine residue (TCC) of the published sequence is replaced with a phenylalanine residue (TTC). Human (*2, 24*) and mouse neurofibromin (*25*) contain a phenylalanine residue at the equivalent positions, favoring the sequence of pRc/RNF. The amino acid identity in the GRD is about 98.9 and 99.7% between human and rat and between rat and mouse, respectively.

The relative expression levels of type I and type II neurofibromin mRNAs were determined in various tissues of adult rat by S1 nuclease mapping analysis, and a representative result is shown in Fig. 2. In this assay, the S1 probe gave rise to protected fragments of about 100 bases with type I mRNA and about 269 bases with type II mRNA, in addition to the 549-bp fragments representing the undigested probe. Type I mRNA was predominantly expressed in every region of the brain examined, pituitary, and testis. Type I and type II mRNAs were equally expressed in spleen and jejunum, and type II mRNA was predominantly expressed in lung, heart, liver, kidney, ovary, and adrenal gland (cortex and medulla) (Fig. 2). Highest expression levels of type I neurofibromin mRNA were observed in the adult testis and brain regions.

The developmental changes of expression of type I and type II mRNAs were studied in the whole brain and testis obtained from 1- to 8-week-old rats (Fig. 3). Type II mRNA was predominant in the testis at 1 week, and type I mRNA became predominant in the testis at 3 weeks. During the subsequent development of the testis, type I mRNA was the predominant form and type II mRNA expression gradually decreased. In contrast, type I mRNA was predominantly expressed in the whole brain throughout the same developmental stage.

DISCUSSION

This is the first report showing the entire structure of rat type I neurofibromin and its mRNA, which will help us identify multiple neurofibromin transcripts in rat, such as amino-terminal isoforms (*11, 12, 26, 27*) found in humans. The present study will also facilitate research on the regulation of expression of neurofibromin isoform mRNAs under pathophysiological conditions, because rat provides suitable *in vivo* models for hemodynamic stress (*28*) and transient forebrain ischemia (*29*). The sequence data are also invaluable for evaluating the functional significance of amino acid substitutions that will be found in neurofibromin of NF1 patients or certain primary tumors. The extremely high homology in the amino acid sequence between rat and human neurofibromin is consistent with our previous report that the antibody raised against the GRD of human type I neurofibromin (*30*) recognizes a protein of over 200 kDa expressed in the cells transiently transfected with a rat type I neurofibromin cDNA pRc/RNF (*9*).

The present study also shows differential tissue-specific expression of type I and type II neurofibromin mRNAs in rat. However, it should be noted that the S1 nuclease mapping analysis allowed us to detect the presence of the transcripts with or without the 63-base insertion but not to discriminate a certain transcript, such as a 3′ alternatively spliced transcript found in humans (*17*), from type I or type II mRNA. It remains to be investigated whether such a 3′ alternatively spliced transcript is expressed in rat tissues.

Type I neurofibromin mRNA was predominantly expressed in the brain regions, pituitary, and testis of adult rats. We previously showed by S1 nuclease mapping analysis that type I mRNA was predominantly expressed in the normal human brain (*26*), which is compatible with the results of the present study in rat brain and with the

findings in chicken brain (*21*). In addition, both types of neurofibromin mRNAs are expressed at similar levels in PC12 rat pheochromocytoma cells, and neuronal differentiation of PC12 cells induced by nerve growth factor was not accompanied by significant changes in the amounts of neurofibromin mRNAs or in the relative levels of type I and type II mRNAs (data not shown). On the other hand, other investigators have shown using the reverse transcription-PCR method that type II mRNA is expressed predominantly in the normal human brain and in a neuroblastoma cell line and an astrocytoma cell line, both of which were induced to differentiate into neural cells by treatment with retinoic acid (*13*). The differences in the results among these reports could be due to the methods employed. Indeed, Andersen *et al.* (*15*) pointed out the difficulty in quantitation of the relative amounts of type I and type II mRNAs by the reverse transcription-PCR method, because some variation was seen even when the same RNA samples were used.

The relative levels of type I and type II mRNAs are changed in the testis during the postnatal development. At 4 to 6 days of age, spermatogenesis starts in most tubules, and cyclic changes of seminiferous epithelium similar to those observed in the adult are established by 2 to 3 weeks (*31*), when type I mRNA becomes the predominant form. During the subsequent developmental period, type I mRNA is expressed at higher levels compared to type II mRNA, as seen in adult animals. In contrast, type I mRNA was predominantly expressed in the whole brain during the same period, as seen in adult brain regions. The relative expression levels of type I and type II mRNAs are therefore specific to each tissue and, at least in the testis, may dynamically change depending on the state of cell differentiation.

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