Expression and assembly of a human neurofilament protein in transgenic mice provide a novel neuronal marking system

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To investigate the regulation of neurofilament gene expression, we have generated several lines of transgenic mice carrying multiple copies of a cloned human neurofilament (NF-L) gene. We show that a 21.5-kb DNA fragment including the human NF-L gene contains essential information for correct expression in nervous tissue of transgenic mice. The integrated genes are arranged in multiple tandem arrays, but the extent of transgene expression does not correlate with copy number nor does it influence the expression of the endogenous neurofilament genes. However, the levels of human NF-L protein recovered in neurofilament preparations from brains of transgenic mice correlate directly with the relative abundance of human NF-L mRNA detected in each line. There is an apparent delay in the accumulation of human NF-L protein during development, as determined by immunoblotting with a human-specific monoclonal antibody. Finally, immunohistochemical localization of the human NF-L protein results in the specific staining of neurons and their processes in transgenic mice.

[*Key Words:* Neurofilament; transgenic mice; tissue-specific expression; developmental gene regulation; neuronal marking system]

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Introduction of cloned DNA into mice by microinjection of fertilized mouse eggs (Gordon et al. 1980) provides a powerful approach to study gene regulation and function. Thus, the use of transgenic mice has provided valuable information on the regulatory elements that control tissue-specific expression in various cell types, including pancreatic (Swift et al. 1984; Hanahan, 1985), erythroid (Magram et al. 1985; Townes et al. 1985; Kollias et al. 1986), lymphoid (Grosschedl 1984; Adams et al. 1985), yolk sac and liver (Krumlauf et al. 1985), mammary (Stewart et al. 1984), eye lenses (Overbeek et al. 1985; Goring et al. 1987), hepatocytes (Kelsey et al. 1987), and oligodendroglial cells (Readhead et al. 1987). However, there have been few reports concerning expression of transgenes in neurons (Mason et al. 1986; Gordon et al. 1987), and none in which expression in the majority of neuronal cell types was observed. The feasibility of introducing a gene expressed in virtually all neurons would offer a unique opportunity to elucidate some of the fundamental mechanisms that underlie gene expression during nervous system development and may provide a means to direct expression of foreign gene products in nervous tissue. Moreover, if the transgene product was detectable *in situ*, it would provide a powerful genotype marking system applicable to both developmental and regeneration studies of the mammalian nervous system.

Neurofilaments, which have been widely used as markers of nerve cells of both the central and peripheral

nervous systems, are formed by the copolymerization of three proteins with apparent molecular weights on SDS-gels of 68,000 (NF-L), 145,000 (NF-M), and 200,000 (NF-H) (Hoffman and Lasek 1975; Liem et al. 1978; Julien and Mushynski 1982). Neurofilament proteins belong to the multigene family of intermediate filament (IF) proteins, which includes vimentin, in cells of mesenchymal origin; keratins, in epithelial cells; desmin, in muscle cells; and the glial acidic fibrillary protein in glial cells (for review, see Lazarides 1982; Osborne and Weber 1982). These IF proteins share a homologous central α -helical domain of conserved length, capable of forming a coiled-coil configuration, which is flanked by amino- and carboxy-terminal regions that are hypervariable in size and composition (Geisler and Weber 1982; Geisler et al. 1984; Hanukoglu and Fuchs 1982; Quax et al. 1983, 1985; Steinert et al. 1983; Julien et al. 1985, 1986). With the exception of keratin filament formation, which requires one member of type I and type II keratins (Lee and Baden 1976; Steinert et al. 1976; Milstone 1981; Franke et al. 1983), all IF proteins are capable of homopolymerization (Geisler and Weber 1981, 1982; Steinert et al. 1981). The protofilament has a tetrameric organization of two coiled-coil dimers (Geisler et al. 1982, 1985; Quinlan et al. 1984; Parry et al. 1985).

The neuronal specificity of neurofilament gene expression has been confirmed using a cloned DNA probe in RNA blot analyses (Julien et al. 1985, 1986) and by *in situ* hybridization studies (Liesi et al. 1986). Initial ex-

pression of neurofilaments is coincident with the appearance of postmitotic neurons and axon extension (Cochard and Paulin 1984; Bennett and DiLullo 1985), but, interestingly, there is a temporal difference in the expression of the three neurofilament genes during development. For the mouse, the NF-L and NF-M genes begin to be expressed in the fetus, but high levels of NF-H gene expression are delayed to the postnatal period (Shaw and Weber 1982; Willard and Simon 1983; Julien et al. 1986). The NF-L, NF-M, and NF-H genes have recently been cloned (Julien et al. 1986, 1987; Lewis and Cowan 1986; Levy et al. 1987; Myers et al. 1987), and transient expression of the human NF-L gene was observed when transfected into mouse L cells (Julien et al. 1987). However, the regulatory elements that control their developmental and tissue-specific expression have not been defined.

We report here that a 21.5-kb DNA fragment bearing the human NF-L gene contains all essential *cis*-regulatory sequences for neuron-specific expression in transgenic mice. Moreover, the human NF-L protein is assembled into neurofilaments and can be detected in neuronal cell bodies and their processes by immunohistochemical procedures.

Results

Genomic hybridization analysis of transgenic mice

A *SalI* DNA fragment of the cosmid pHNFL that contains the human NF-L gene (Julien et al. 1987) was microinjected into the male pronucleus of fertilized mouse eggs. The restriction map in Figure 1 shows that this 21.5-kb DNA fragment includes, in addition to all exon

sequences, 14 kb of 5'-flanking sequences and 3.2 kb downstream of the first polyadenylation site.

The presence of human NF-L DNA in offspring of founder mice was first determined by Southern blot analysis of DNA extracted from the tails. Hybridization with a *SmaI*–*PvuII* probe, which spans the first exon of the NF-L gene, was used for detection of a 6.5-kb *EcoRI* fragment unique to human DNA (data not shown). Of 50 offspring derived from pronuclear injected eggs, 7 contained the diagnostic fragment. The copy number of the human NF-L gene was estimated in the offspring of founder mice by slot-blot hybridization using 10 µg of tail DNA (Fig. 2, left lane). As a copy standard, a plasmid containing the human NF-L gene was mixed with 10 µg of mouse DNA (Fig. 2, right lane). To minimize cross-hybridization with the endogenous mouse NF-L gene, hybridization was carried out at high stringency with a 3.2-kb *EcoRI*–*SalI* DNA probe corresponding to the 3' untranslated region of human NF-L gene. There is considerable variation in the number of copies from one mouse line to another. Densitometric scanning of the autoradiograph in Figure 2 indicated that only three copies of the human NF-L gene integrated in transgenic line 27, whereas lines 41, 42, and 29 contain over 200 copies.

From all of the transgenic lines, an 8.7-kb fragment of high intensity is revealed in *EcoRI*-digested tail DNA hybridized with the 3' *EcoRI*–*SalI* human probe. This is the predicted size of the junction fragment anticipated if the multiple copies of the human NF-L DNA were integrated in a head-to-tail tandem array (data not shown). In transgenic lines 27 and 32, unique bands of weaker intensity can be detected, which most likely represent junctional fragments at each insertion site. In lines 29,

Figure 1. Restriction cleavage map of the injected human NF-L DNA fragment. Abbreviations of restriction enzymes: (B) *Bam*HI; (Bg) *Bgl*II; (C) *Cla*I; (E) *Eco*RI; (P) *Pvu*II; (Sm) *Sma*I; (S) *Sal*I; (X) *Xho*I. A schematic representation of the NF-L gene depicts exons as boxes. The diagram at the bottom illustrates the interpretation of the RNase-protected fragment of the subcloned region of NF-L gene.

Figure 2. Slot-blot hybridization of genomic DNA showing amounts of integrated human NF-L genes in six transgenic lines. Tail DNA (10 µg) samples from the offspring of six transgenic founders were transferred to nitrocellulose and hybridized with an *EcoRI-Sall* probe corresponding to 3'-untranslated sequences of the human NF-L gene to minimize cross-hybridization with the endogenous mouse NF-L gene (left lane). The number of integrated gene copies was estimated by hybridizing different amounts of a plasmid DNA containing the human NF-L gene mixed with 10 µg of wild-type mouse DNA (right lane).

34, 41, and 42, the precise identification of junctional fragments is hindered by the high copy number of the integrated human NF-L gene. However, in these lines, the copy number remains constant in subsequent generations, and, as expected for single loci, 50% of the offspring from hemizygotes mated to normal mice are transgenic.

Expression of human NF-L mRNA transgenic mice

The offspring of six founder mice were examined for the presence of human NF-L transcripts in their brain RNA. To distinguish between human and endogenous mouse NF-L transcripts, RNA samples were assayed by a sensitive RNase protection assay (Melton et al. 1984), using an anti-sense RNA probe derived from a 958-bp *Bgl*III fragment of the human NF-L gene, as illustrated in Figure 1. Following hybridization of the ³²P-labeled probe with human brain RNA, a 316-nucleotide fragment corresponding to the first exon sequences is protected from RNase digestion (Fig. 3, lane 3). Mouse brain RNA yields RNase-resistant products of approximately 150 nucleotides (Fig. 3, lane 2) but at much lower radioactive intensity. In this protection assay the band intensity is dependent upon the length of protected fragments, the stability of hybrid molecules, and nucleotide content.

As shown in Figure 3, human NF-L mRNAs were detected in brain RNA from five of the six transgenic mice examined, and in four lines the detected levels of human NF-L transcript approximate that found in human brain RNA. However, the levels observed do not correlate with transgene copy number. Mice 29-43, 34-5, and 41-10 all contain more than 200 transgene copies, whereas mouse

27-8 contains only 3. Barely detectable amounts of human NF-L mRNA were found in mouse 32-2, which contains 15 gene copies, whereas no expression was detected in mouse 42-8, which carries 200 copies of human NF-L gene. It should be noted that the presence of sev-

Figure 3. Expression of human NF-L gene in the brain of transgenic mice. Total brain RNAs (10 µg) from various F₁ hemizygous transgenic mice were examined for the presence of human NF-L mRNA by a sensitive RNase protection assay (Melton et al. 1984). The RNA probe specific to human NF-L gene is depicted in Fig. 1. Following RNase treatment of human brain RNA (10 µg), a 316-nucleotide sequence is protected (lane 3), whereas resistant fragments of ~150 nucleotides are generated with brain RNAs (10 µg) from normal mice (lane 2). High-level expression of the human NF-L is observed in four of the six transgenic mice (lane 4-9). Markers are end-labeled *Hinf*I fragments of pBR322 (lanes 1 and 10).

eral copies of the human NF-L gene in the various transgenic mice did not appear to affect the expression of the endogenous mouse NF-L gene.

An S1 nuclease protection assay indicated correct initiation of transcription of the human NF-L gene in mice 27-8 and 29-43 (data not shown). Southern blot analysis of all six lines revealed no evidence of rearrangements involving the cloned DNA that was microinjected into the zygotes (data not shown).

Tissue-specific expression of the human NF-L transgene

To examine whether the human NF-L gene is expressed in a tissue-specific manner in transgenic mice, RNase protection assays were carried out on RNA samples obtained from different tissues of mature mice (>2 months) from lines 27 and 29. Figure 4 shows that in mouse 29-43, human NF-L mRNA was detected only in RNA from the brain, whereas RNA samples from the spleen, intestine, muscle, liver, heart, and kidney revealed no detectable human or mouse NF-L mRNA. Tissue-specific expression was also observed with mouse 27-8 (data not shown).

Synthesis and assembly of human NF-L protein

Neurofilament-enriched fractions were prepared from human brain and from brain stem of normal and transgenic mice by the axon flotation procedure described previously (Julien and Mushynski 1981). As shown on the Coomassie-blue-stained gel in Figure 5, the three neurofilament proteins, NF-L, NF-M, and NF-H, constitute prominent components in these preparations. Between human and mouse, marked differences are observed in electrophoretic migration of the NF-M proteins. However, the apparent molecular weights of human and mouse NF-L proteins are similar; therefore, in gels it is not possible to distinguish the foreign protein in neurofilament preparations of transgenic mice. To identify the human NF-L protein, we used a monoclonal antibody raised against bovine NF-L, which recognizes the human but not the mouse NF-L protein. The immunoblot in Figure 5 illustrates the specificity of this monoclonal antibody. A strong signal is observed with human NF-L protein (Fig. 5, lane 1), but no cross-reaction is detected with the corresponding mouse protein (Fig. 5, lane 2). The epitope on the human NF-L protein, recognized by the antibody, has not been identified, but a sequence comparison between human (Julien et al. 1987) and mouse NF-L protein (Julien et al. 1986; Lewis and Cowan 1986) indicates substantial amino acid divergence (10%) in the carboxy-terminal regions.

Using the immunoblot procedure, human NF-L protein was identified in both brain homogenates and in assembled neurofilaments prepared from myelinated axons of transgenic mice 27-8 and 29-43 (Fig. 5, lanes 3,4,6, and 7). Based on visual analysis of the immunoblot assay, we estimate that human NF-L protein constitutes 10% and 30% of total NF-L protein assembled in axonal

neurofilaments of mice 27-8 and 29-43, respectively. The relative proportion of human NF-L protein detected in these two transgenic mice is equivalent to the relative human NF-L mRNA concentrations observed in these two lines (Fig. 3, lanes 4 and 9).

Developmental expression of human NF-L protein is delayed in transgenic mice

The timing of human NF-L protein expression was examined during brain development of the transgenic mouse line 29. Hemizygous male transgenic mice of line

Figure 4. Tissue-specific expression of human NF-L mRNA in transgenic mice. RNA samples (10 μ g) prepared from various tissues of transgenic mouse 29-43 (lanes 4-10) were analyzed for the presence of human NF-L transcripts by RNase protection assays.

Figure 5. Immunodetection of human NF-L protein in transgenic mice. Neurofilament-enriched fractions from myelinated axons (NF) (lanes 1–4) and protein from whole brain homogenates (H) (lanes 5–7) were analyzed by SDS–gel electrophoresis, and the gel stained with Coomassie blue (left panel). An identical gel was run in parallel and used for immune blotting with a specific monoclonal antibody that recognizes human NF-L protein (right panel). Following reaction with ^{125}I -labeled protein, the bands were visualized by autoradiography.

29 were mated to BALB/c females, and the fetus or offspring carrying the transgene locus were identified by immunoblotting whole brain proteins, as described above.

An antibody that recognizes both human and mouse NF-L proteins reveals a progressive increase of NF-L expression in both normal and transgenic mice during brain development (Fig. 6A,B). These results are similar to the developmental profile of mouse NF-L mRNA expression reported previously (Julien et al. 1986). However, expression of human NF-L protein, as determined with the human-specific monoclonal antibody, is delayed during development with high levels detected only in the postnatal brain (Fig. 6C).

Immunohistochemical detection of the human NF-L protein in neuronal cell bodies and their projections

Spinal cords and brains from line-29 transgenic and normal mice were mounted together in pairs prior to freezing. Cryostat sections of these fixed frozen preparations revealed equivalent and high levels of antibody binding to axons when reacted with monoclonal antibody RT97 (Anderton et al. 1982), an antibody recognizing a phosphorylated epitope prominent on mouse NF-H protein present in neuronal processes. Only the transgenic nervous tissue revealed antibody binding when incubated with the monoclonal recognizing the human NF-L protein (results for the spinal cord shown in Fig. 7). No labeling was observed on control sections

similarly processed with the omission of primary antibody, indicating that all procedures and reagents used result in specific labeling. For the spinal cord sample, the anti-human NF-L antibody bound to the majority of,

Figure 6. NF-L protein expression during brain development. Protein samples (100 μg) from whole brain of mice at different developmental stages were analyzed by immune blotting for the presence of NF-L proteins. The different lanes represent immunodetection of NF-L proteins at embryonic days 15 and 18, and postnatal days 1, 7, 15, and adult. An anti-NF-L antibody that recognizes both mouse and human NF-L protein was used to examine NF-L protein expression during brain development of normal mice (A) and hemizygous transgenic mice (B). The human NF-L protein in the same transgenic mouse samples was detected with an anti-human NF-L antibody that does not recognize the corresponding mouse protein (C).

if not all, axon profiles and some neuronal cell bodies were stained intensely. In contrast, sections of the brains from these transgenic mice revealed prominent human NF-L labeling only in neuronal processes, whereas cell bodies appeared unstained. However, analysis of adjacent brain sections revealed that all fiber tracks immunolabeled with RT97 were similarly labeled with anti-human NF-L antibody (Fig. 8). Thus, human NF-L expression was observed only in neurons and their processes, whereas all other cell types present in the central nervous system, including glia, fail to express detectable levels of the human NF-L protein.

Discussion

Expression of the human NF-L gene was observed in the brain of five of six lines of transgenic mice and is strictly limited to nervous tissue. Although the various chromatin domains into which the human NF-L gene integrated in each transgenic line could result in differences in transcriptional activity, these results indicate that *cis*-regulatory elements in the human NF-L gene respond to factors controlling neuronal-specific expression of the endogenous mouse NF-L gene. Transgene expression has been described for nonneuronal genes, such as these encoding elastase (Swift et al. 1984), immunoglobulins (Grosschedl et al. 1984), α -fetoprotein (Krumlauf et al. 1985), collagen (Khillan et al. 1986), myosin (Shani 1985), globin (Magram et al. 1985; Townes et al. 1985; Kollias et al. 1986), insulin (Hanahan 1985), α - and γ -crystallins (Overbeek et al. 1985; Goring et al. 1987), α_1 -antitrypsin (Kelsey et al. 1987), and myelin basic protein (Readhead et al. 1987). A general conclusion from these studies is that *cis*-regulatory sequences are involved in the appropriate expression of these genes, and our observations with the neuronal specific gene described here are consistent with this view. However, there may be species differences in signals involved in the control of NF-L gene expression, as the apparent developmental

profile of the human NF-L transgene does not strictly parallel that of the mouse NF-L gene. It is possible that the microinjected DNA fragment was missing some sequences required for developmental stage-specific expression. Alternatively, we assessed the developmental expression of the human NF-L transgene by the level of

Figure 7. Immunohistochemistry of neurofilament proteins in spinal cords of normal and human NF-L transgenic (line 29) mice. Spinal cords from mature mice (>2 months) were mounted together with ventral surfaces opposing and cross sectioned as a single sample. (A) Hematoxylin and eosin stain. Transgenic cord is on the left, and normal cord is on the right, bisected by the ventral fissure. (B) Reaction with anti-NF-H antibody RT97, which recognizes a phosphorylated epitope prominent in assembled neurofilaments in axons of the mouse. (C) Reaction with NF-L antibody, which recognizes the human but not the mouse NF-L protein. Profiles of axons, as well as some neuronal cell bodies, are intensely labeled in the transgenic spinal cord, whereas no similar labeling is detected in the normal spinal cord. Bar represents 20 μ m.

Figure 8. Coronal sections of brain from line-29 transgenic mouse reacted with anti-mouse NF-H RT97 (left column) and anti-human NF-L (right column). (A,B) Low-magnification photomicrographs revealing the prominent fiber tracks in hippocampus. Cerebral cortex is on the right of each image. All tracks are labeled with both antibodies, and the staining intensity appears to be equivalent in the majority. Final magnification, $\sim 95\times$. (C,D) Tapetum, cerebral cortex (right), and Alveus hippocampi (left). No labeling is observed over cell bodies. Magnification, $375\times$. (E,F) Basal optic tract. Hippocampus on bottom of image. Magnification, $375\times$. (G,H) Border between basal optic tract and hippocampus. Only fibers are labeled. Magnification, $1500\times$.

Figure 8. (See facing page for legend.)

immunoreactive material observed on Western blots. If the epitope recognized by the antibody involved post-translational modification of the NF-L protein, the apparent developmental delay might not reflect gene activation or mRNA expression. Rather, it may involve exclusively the developmental profile of a post-translational modification system. Regardless of the mechanism underlying the observed developmental profile, it remains to be determined whether all neurons share an identical phenotype or whether there are subpopulations of developing neurons expressing different levels of human NF-L.

The relative inactivity of the transgene complex observed in lines 32 and 42 might be explained by the particular integration sites in these two lines. Among the four lines where expression was detected, generally higher levels of human NF-L mRNA were observed in transgenic mice with a high copy number (>200 copies per cell), but there was no linear correlation between mRNA expression and the gene copy number. The mechanism underlying this result is not clear. If the concentration of specific transcription factors was strictly rate limiting, the high copy number of human NF-L transgenes that exist in some of these lines could result in a dilution effect, causing a relevant decrease in the expression levels of the endogenous mouse NF-L gene. Similarly, if a post-transcriptional mechanism regulating the stability of all NF-L mRNA was involved, the transcript derived from the mouse gene should be subjected to the same influence. However, the detected level of endogenous mouse NF-L mRNA was apparently unaltered in all of the transgenic mice examined.

The general failure to observe gene dosage effects for the expression of the human NF-L transgene loci examined suggests that additional levels of regulation remain distinct possibilities. One such possibility is transcriptional interference between adjacent genes integrated in tandem head-to-tail arrays. Such transcriptional interference has been described previously in expression assays with constructs containing adjacent α -globin genes introduced into HeLa cells (Proudfoot 1986). These studies demonstrated substantial inhibition of the downstream α -globin gene by transcription of the corresponding upstream gene. This apparent inhibition appears to be caused by RNA polymerase II molecules that transcribe the 5' gene with subsequent readthrough of the 3' adjacent gene, thus preventing independent initiation of transcription in the downstream gene. By analogy, it is possible that only a small fraction of the NF-L genes arranged in tandem are transcriptionally active in the transgenic mice described here. An alternate possibility is that the transgene loci have integrated into regions of the genome in which genome imprinting occurs. Recently, variation in the methylation status of transgene loci has been observed, and, in a proportion of transgenic lines all containing the same construct, modification of the transgene methylation status occurs in a gamete-specific fashion during its inheritance. Although the full consequences of this modification remain to be elucidated, these results are consistent with the mechanistic requirements of differential genome expression under-

lying the phenomenon of genome imprinting. Regardless of the mechanism, integration of the transgene into such an imprinted region may affect the level of expression in primary transgenics and may also affect expression in subsequent generations (Reik et al. 1987; Sapienza et al. 1987).

The human NF-L transcripts produced in mice 27-8 and 29-43 are correctly processed and translated into functional proteins. In these two transgenic mice, the levels of foreign protein detected in total brain homogenates, as well as in neurofilament preparations, parallel the levels of mRNA expressed. Therefore, an increased synthesis of human NF-L proteins leads to a corresponding increase in proteins assembled into axonal filaments. Neither the presence of several copies of NF-L gene or increased abundance of foreign NF-L protein in mouse 29-43 caused repression or induction in synthesis of any of the three endogenous mouse neurofilament proteins. Thus, the synthesis and assembly of human NF-L protein in transgenic mice resemble the expression of foreign keratins in epithelial PtK₂ cells in which proteins, produced after mRNA injection (Franke et al. 1984) or DNA transfection (Giudice and Fuchs 1987), simply copolymerize with the endogenous IF network. In contrast, forced expression of a type-II keratin in fibroblasts was found to induce type-I keratin expression, and both types are prerequisites for the formation of filamentous structures (Giudice and Fuchs 1987).

The immunohistochemical results are fully consistent with neuron-specific expression of the transgene. The patterns of immunolabeling observed in brain suggest that the particular epitope recognized by the anti-human NF-L antibody may be most prominent in the assembled neurofilaments present in neuronal processes, as is the case with the phosphorylated epitope of NF-H recognized by RT-97. The presence of labeling in some neuronal cell bodies in the spinal cord could reflect an exceptional localization of fully modified and assembled neurofilaments.

The stable inheritance of the human NF-L transgene and the neuron-specific expression of a nondiffusible cytoskeletal component that is distinguishable *in situ* with a monoclonal antibody should provide the basis of an immunohistochemical marking system to study neuronal development in chimeras and to investigate both cell survival and axonal elongation in nerve grafting experiments. Our results suggest the further possibility of directing foreign protein expression exclusively to nerve cells in transgenic mice with the use of hybrid genes containing NF-L regulatory regions. Analysis of deletion mutants and chimeric constructs involving the NF-L gene is now in progress and should provide valuable information on the identity of regulatory elements that control expression.

Materials and methods

Microinjection into mouse eggs and DNA blot analysis

A 21.5-kb *SalI* fragment of the human NF-L gene (Julien et al. 1987) was injected at 2 μ g/ml into the male pronuclei of

C3B6F1 $\times$ B6D2F1 fertilized eggs. Injected eggs were transferred to the oviduct of pseudopregnant females (Brinster et al. 1985). Identification of transgenic mice was carried out by Southern blot analysis of tail genomic DNA. DNA (10 μ g) was digested with *EcoRI*, fractionated on 0.7% agarose gels, and transferred to nitrocellulose. The blots were hybridized with a *SmaI*-*PvuII* 32 P-labeled fragment of the human NF-L gene (Julien et al. 1987) for detection of a 6.5-kb band unique to human. The offspring of founder mice were analyzed by slot-blot hybridization with 10 μ g of tail DNA using an *EcoRI*-*SaII* fragment corresponding to the 3'-untranslated region of the human NF-L gene to minimize cross hybridization with the corresponding mouse NF-L gene.

RNase protection assays

RNA was prepared from various tissues by the guanidinium/CsCl method (Maniatis 1982). A 958-bp *BglIII* fragment containing exon I and intron I sequences of the human NF-L gene was subcloned into the pT7/T3-18 vector (BRL). After linearization of the plasmid with *HindIII*, an anti-sense RNA probe of high specific radioactivity was generated with T7 RNA polymerase in the presence of [α - 32 P]CTP, according to instructions of the manufacturer. An excess of RNA probe was hybridized at 45°C with 10 μ g of total RNA, and RNase treatment was carried out at 34°C as described previously (Melton et al. 1984). RNase-protected fragments were resolved on denaturing 5% polyacrylamide gels. 32 P-labeled fragments of pBR322 plasmid digested with *HinfI* served as markers.

Neurofilament preparation

Myelinated axons were prepared from the brainstems of mice by a flotation procedure described previously (Julien and Mushynski 1981) and were lysed by homogenization in 1% (wt/vol) Triton X-100, 0.01 M sodium phosphate (pH 6.5). The neurofilament-enriched fractions were obtained by centrifugation for 1 hr at 27,000g through 0.85 M sucrose containing the same buffer.

Immune blotting

Protein samples were solubilized in SDS sample buffer and electrophoresed on SDS-8.5% polyacrylamide gel. For immune blotting, the proteins were transferred electrophoretically to nitrocellulose membranes and incubated with either a monoclonal antibody raised against bovine NF-L (clone DP5-112) that also recognizes human NF-L (Daymar Laboratories, Toronto) or with a monoclonal antibody (Amersham) that recognizes the NF-L protein from both human and mouse. The antibodies were detected by incubation with 125 I-labeled protein A (80 μ Ci/ μ g) or 125 I-labeled anti-mouse IgG (Amersham), and bands were visualized by autoradiography.

Immunohistochemical preparations

Mice were anesthetized with avertin and perfused via cardiac puncture, first with 0.1 M phosphate buffer (pH 7.2) followed by 4% paraformaldehyde, freshly prepared in the same buffer. Perfusion in fixative was continued for 15 min, and dissected tissues were placed in fixative for an additional 1–2 hr (all at room temperature), prior to returning tissues to buffer alone at 4°C for 16 hr. Tissues from both normal and transgenic mice were mounted together on cork with mouse liver, frozen in liquid-nitrogen-cooled isopentane, and stored at -135°C . Cryostat sections (6 μ m thick) were picked up on polylysine-

coated slides and incubated at room temperature as follows: 10 min in 0.1 M Tris-HCl (pH 7.6); 30 min in 4% paraformaldehyde; 0.1 M phosphate; 0.5 M NaCl (pH 7.3); 3 $\times$ 10 min in 0.1 M Tris-HCl (pH 7.6); 15 min in 0.01 M glycine in 0.1 M Tris-HCl (pH 7.6); 3 $\times$ 5 min in 0.1 M Tris-HCl (pH 7.6); and overnight in 1 : 100 (anti-human NF-L) or 1 : 500 (RT97) dilutions of primary antibody prepared in 0.1 M Tris (pH 7.6) containing 1% normal goat serum. Control slides were processed similarly with no addition of primary antibody. Antibody was detected with biotinylated anti-mouse IgG, followed by avidin-biotin peroxidase complex (ABC), as per manufacturer's (Vector Labs) instructions.

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