Multiple Neuron-specific Enhancers in the Gene Coding for the Human Neurofilament Light Chain*

(Received for publication, June 29, 1995, and in revised form, October 27, 1995)

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To define DNA regions involved in the neuron-specific expression of the neurofilament light (NF-L) gene, we generated transgenic mice bearing different NF-L constructs. A 4.9-kilobase human NF-L fragment including –292 base pairs of 5’-flanking sequences contained sufficient elements for nervous system expression in transgenic mice. Deletion of introns 1 and 2 from this 4.9-kilobase DNA fragment resulted in reduced levels of transgene expression in the cortex, while deletion of intron 3 had little effect. Both introns 1 and 2 could act independently as enhancers to confer neuronal expression of either the lacZ reporter gene or an intronless hNF-L construct. Sequence comparison revealed that intron 1, intron 2, and the basal human NF-L promoter all contain an ETS-like motif, CAGGA, present in a variety of genes expressed in the nervous system.

Neurofilaments are formed by the copolymerization of three neuron-specific proteins with an apparent molecular weight of 70 kDa (NF-L), 150 kDa (NF-M), and 200 kDa (NF-H) (1–3). The genes coding for the three NF1 proteins have been cloned and sequenced (4–10). Like other intermediate filament (IF) genes, NF genes are expressed in a cell type-specific and developmental manner (11–13). IF genes, from class IV and VI, are differentially regulated during the development of neuronal progenitor cells (5, 9, 14–16).

Neurofilaments are expressed in most neurons of the nervous system, and their expression coincides with terminal neuronal differentiation. There is growing evidence that deregulation of neurofilament expression may play a central role in motor neuron disease. Transgenic mice that overexpress neurofilament proteins show motor neuron degeneration (17–21). IF genes, from class IV and VI, are differentially regulated during the development of neuronal progenitor cells (5, 9, 14–16).

Motor neuron disease. Transgenic mice that overexpress neurofilament proteins show motor neuron degeneration (17–21). Furthermore, the levels of NF-L mRNA are decreased in dogs with hereditary canine spinal muscular atrophy (22) as well as in motor neurons of patients with amyotrophic lateral sclerosis (23). Yet, little is known about the mechanisms that regulate expression of neurofilament genes in the nervous system. This is in part due to the lack of suitable in vitro systems to study their expression (15). For instance, high levels of NF-L expression occurred after transfection of a complete genomic NF-L gene in non-neuronal cells such as cultured fibroblasts (6, 24), even though the endogenous NF-L gene remained silent. In contrast, DNA fragments containing either the complete human or mouse NF-L genes were correctly expressed in transgenic mice (25, 26).

We have shown previously that a human NF-L fragment including –292 bp of 5’-flanking sequences and intron sequences contained sufficient elements to drive NF-L expression in the nervous tissues of adult transgenic mice (27). To further clarify the potential role of introns in modulating expression of the human NF-L gene, we used the transgenic mouse approach to test the transcriptional activity of NF-L DNA regions. We report here the existence of neuron-specific enhancers in both introns 1 and 2, which contribute to a wider expression pattern of the NF-L gene. In addition, we show that the regulatory elements included in the NF-L basal promoter (–292 bp) are sufficient to target neuronal expression.

EXPERIMENTAL PROCEDURES

Plasmid Construct—To delete introns of the hNF-L gene, we took advantage of the existence of conserved restriction sites between the human and mouse NF-L genes. First, a 4.9-kb BamHI-XbaI fragment including the human NF-L gene was subcloned into the corresponding sites of a Bluescript pSK+ vector giving plasmid pSKhNF-L (27). The Pshl–NF-L plasmid was subjected to a total BgIII digestion followed by a partial SacI digestion, resulting in deletion of a DNA fragment spanning intron 1 and 2 all together with part of exon 1 and 3. The remaining plasmid pSKhNF-L was then isolated on agarose gel. A corresponding DNA BgIII–SacI fragment of 750 bp from the mouse cDNA (5) was subcloned in that vector to recreate a human–mouse hybrid NF-L gene with its complete coding sequences but lacking introns 1 and 2 (construct hNF-L/Intron3). A similar approach was used to delete intron 3, except that a double SacI–EcoRI partial digestion was carried out. The 522-bp SacI–EcoRI fragment from the mouse cDNA was used to replace the third intron. For the hNF-L/Intronless and the intronless-UTR, the pSKhNF-L was digested to completion by BgIII followed by a partial EcoRI digestion. From the mouse cDNA clone 646 (5, 13), a BgIII–EcoRI fragment of 1200 bp was isolated and ligated to the open pSKhNF-L vector. Prior to microinjection, all deletion mutant constructs were isolated on agarose gel after complete BamHI–XbaI digestion except for the intronless-UTR, the DNA fragment was isolated after enzymatic digestion with BamHI–PstI. The PstI restriction site is localized 35 bp after the first polyadenylation signal of the human NF-L gene.

The β-galactosidase gene (lacZ) was obtained from plasmid pCH110 (Pharmacia Biotech Inc.). The pCH110 was cut to completion with HindIII followed by EcoRI partial digestion. A fragment of 4486 bp was then isolated and subcloned into Bluescript pSK+ linearized with HindIII–EcoRI digestion, creating plasmid pSKlacZ. The human NF-L basal promoter (from –292 to +15 bp) was isolated, blunt-end ligated to HindIII linker prior to insertion into HindIII site in pSKlacZ giving hNF-L/lacZ construct. A XhoI-NdeI DNA fragment of 4.8 kb was isolated on agarose gel for microinjection.

* This work was supported in part by the Medical Research Council of Canada and the Canadian Network of Center of Excellence in Neuroscience. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) X05608.

The abbreviations used are: NF, neurofilament; NF-L, neurofilament light; bp, base pair(s); kb, kilobase(s); hNF-L, human neurofilament light; UTR, untranslated region; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; HS, hypersensitive sites; PBS, phosphate-buffered saline.
To prepare the hsp-lacZ construct, we subcloned a lacZ enhancer trap vector (28) into the BamHI sites of a Bluescript SK + plasmid (Stratagene). Each intron was then amplified by PCR and inserted into the unique Smal site localized at the 5′-end of the cassette. For microinjection, each construct was separated from plasmid sequences on agarose gel after digestion with SalI-NsiI.

Microinjection into One-cell Mouse Embryos—Linear DNA fragments were isolated and diluted to concentration of 1 μg/ml in Tris-HCl, pH 7.5, 0.15 M EDTA prior to microinjection into male pronuclei of C57BL/6j X C3H fertilized eggs. Injected embryos were transferred to the oviduct of pseudopregnant females (29). Identification of transgenic mice was carried out by Southern blot analysis of 10 μg of tail genomic DNA as described before (27, 30).

RNase Protection Assay—RNA was prepared from various tissues of transgenic mice by the guanidinium isothiocynate method (31). A 958-bp BglII fragment spanning exon 1 and intron 1 sequences of the human NF-L gene was subcloned into the pT7/T3-18 vector (Life Technologies, Inc.). After linearization of the plasmid with HindIII, an antisense RNA probe of high specific radioactivity was generated with T7 RNA polymerase in the presence of [α-32P]CTP. After 6 h of incubation at 37°C, 2 μCi of 32P-labeled RNA was hybridized to 5 μg of murine cortical RNA at 45°C for 24 h. For RNase protection assay, the transgene (hNF-L) was replaced by the corresponding mouse sequences. Therefore, to determine the specific contribution of intragenic sequences on transcriptional regulation of the hNF-L gene, we generated transgenic mice with various DNA constructs in which introns were deleted in various combinations (Fig. 1). The presence, in exon sequences, of conserved restriction sites between the human and mouse NF-L genes allowed us to selectively swap genomic regions of the human NF-L gene with fragments of the mouse NF-L cDNA. All deletion mutants included the basal human NF-L promoter (−292 bp) and the first endogenous hNF-L polyadenylation signal.

Four transgenic mouse lines have been generated with the intact 4.9-kb hNF-L gene (Fig. 2). In only one mouse line, the transgene remained silent in all tissues examined. In the other three mouse lines, expression of the hNF-L transgene, as detected by RNase protection assay, was restricted to the nervous system. Fig. 3A shows the results of a RNase protection experiment carried out with RNA from an adult mouse of line 1 using an antisense RNA probe covering the first hNF-L exon/intron border.

With the hNF-L/intron1,2 transgene, two out of three mouse lines expressed the transgene (Fig. 2). RNase protection analysis of the RNA from offspring revealed, in both mouse lines, a restricted expression to nervous system (Fig. 3B). In one transgenic line, ectopic expression occurred in the heart (Fig. 2).

For analysis of the hNF-L/intron3, hNF-L/intronless, and the intronless/UTR− constructs, the 960-bp human RNA probe could not be used since the human exon 1 sequences have been replaced by the corresponding mouse sequences. Therefore, to
analyze transgene expression, we carried out an RT-PCR assay with a set of oligonucleotides specific for the 5'-part of the hNF-L first exon (Fig. 4A). The corresponding endogenous NF-L mRNA is not amplified with these primers (Fig. 4B, lane 1) because of species differences in the 5'-untranslated sequences. For the hNF-L/intron3, hNF-L/intronless, and the intronless-UTR, a total of nine different transgenic mouse lines were produced, of which seven expressed their transgene (Fig. 2). All three DNA constructs yielded a restricted expression to nervous tissue. Only line 12, generated with the hNF-L/intronless construct, showed a signal in the heart (Fig. 4B, lane 30).

The removal of introns 1 and 2 resulted in decreased levels of transgene expression in the cortex and cerebellum as detected by RT-PCR (Fig. 4A, compare lane 12 with lane 2). Further deletion of intron 3 and 3'-UTR had little effect on expression pattern and intensity of the bands (Fig. 4B). These results suggested that introns 1 and 2 do contain regulatory elements that contribute to the enhancement of hNF-L expression in the cortex and cerebellum of adult mouse.

Neuron-specific Expression of a lacZ Reporter Gene Driven by the hNF-L Basal Promoter—The above experiment with the hNF-L/intronless construct was capable to direct neuron-specific expression in both embryos and adult mice.

Neuron-specific Enhancers in Human NF-L Introns—The data presented above suggested the existence of regulatory elements within introns of the human NF-L gene. To further confirm the presence of regulatory elements in introns, the DNA fragments spanning intron sequences were tested for their activity using a hsp68/lacZ enhancer trap vector. This vector is composed of the basal promoter of the gene for the heat shock protein hsp68, followed by the E. coli β-galactosidase gene. The minimal hsp68 promoter exhibited no basal activity, indicative of lacZ expression, was found predominantly in the nervous system (Fig. 5). We noted a slight variability in the expression patterns from one embryo to another (Fig. 5), likely reflecting an influence of the chromosomal integration site. This phenomenon, which has been observed for other transgenes (33, 34), can result from the absence of dominant control elements that confer position-independent expression like those found in the β-globin gene (35). Microscopic analysis performed on adult brain sections of line 1 showed expression to diverse population of neurons (Fig. 6). In the cortex, lacZ staining was localized in the forelimb and hindlimb area of the cortex (Fig. 6A) in neurons of the cingulum (Fig. 6C), the lateral septal (Fig. 6D), the piriform cortex (Fig. 6E), and in the hippocampus (Fig. 6F). Strong staining was found in neurons of the granule cell layer of the cerebellum (Fig. 6G). Some neurons of the molecular layer were colored, but no Purkinje cells were stained. lacZ expression was found in the dorsal horn region of the spinal cord and in the dorsal root ganglia (Fig. 6, H and I, respectively). In all regions of the nervous system, only a subset of neurons was stained. The combined results indicate that the basal hNF-L promoter does contain transcriptional elements capable to direct neuron-specific expression in both embryos and adult mice.
activity in transgenic mice (36) unless a heterologous enhancer was placed in its vicinity (28, 37, 38). Thus, the transcriptional activation of the basal hsp promoter reflects the activity of elements carried by a heterologous enhancer. The hNF-L introns 1, 2, and 3 were amplified by PCR and subcloned independently in front of the hsp68/lacZ enhancer trap vector to yield constructs, designated int-1, int-2, and int-3, respectively.

Whole mount transgenic embryos were analyzed for expression of those constructs at 13.5 days post-fertilization. With the int-1 construct, four distinct founders were generated. All four lines expressed the lacZ transgene in the nervous tissue (Fig. 5). A mild variation in the pattern of nervous system expression was detected from one line to another. One embryo showed ectopic expression in the limb buds, while another yielded a very weak signal in the somites. Similarly, the five transgenic embryos bearing the int-2 construct presented a variegated staining in the nervous system. Only one embryo revealed ectopic expression to the apical ectoderm ridge. Of the six transgenic embryos generated with the int-3 construct, none yielded lacZ expression in the nervous system. Ectopic expression was detected at low levels in three lines (Fig. 5).

These results demonstrated the existence of neuron-specific enhancers located in the first and second NF-L introns that can contribute to enhance NF-L expression in the telencephalon, diencephalon, and mesencephalon that correspond to anterior structure of the embryonic nervous system (Fig. 5). The third intron is apparently devoid of such elements. To further define enhancer regions in the first intron, we generated a construct named 5'-int-1 (Fig. 5), in which the first 560-bp region of intron 1 was subcloned in front of the hsp68/lacZ gene construct in an opposite orientation (Fig. 5). Two transgenic embryos were obtained, and they both expressed lacZ in the nervous system. Our conclusion is that the 5'-region of intron 1 contains elements to enhance expression in the anterior region of the nervous system.

**DISCUSSION**

We report here that the minimal human NF-L promoter (−292 bp) is sufficient to direct neuronal expression both of an hNF-L/intronless and an hNF-L/lacZ reporter gene (Figs. 4 and 5). Although a variegated staining occurred in transgenic embryos generated with the hNF-L/lacZ transgene, the lacZ activity was limited to the nervous tissue. This variegation is likely due to an influence of the chromosomal integration site, as chromatin assembly and/or neighboring regulatory elements could affect transcriptional activity. The neuronal expression of the hNF-L/lacZ fusion construct was an unexpected finding, as this same promoter was previously found to be unable to direct neuron-specific expression of the chloramphenicol acetyltransferase reporter gene (27). How could different reporter genes yield different results? It is well known that folding of the DNA in a nucleosomal structure generally decreases basal transcrip-
tional activity (for review, see Ref. 39). Elements capable to initiate loop domain formation, such as matrix attachment regions, can alleviate this phenomenon. We recently demonstrated the presence of matrix attachment regions with DNA unwinding element in the 3′-untranslated region of the hNF-L and lacZ genes but not in the chloramphenicol acetyltransferase gene (40). Following deletion of matrix attachment region sequences from the lacZ gene, the hNF-L/lacZ construct became susceptible to position effect, and its expression was no longer tissue specific (40).

The removal of introns 1 and 2 in the human NF-L gene resulted in a decrease of transgene expression in the cortex and cerebellum of adult mouse. The presence of regulatory elements in introns 1 and 2 was further confirmed using the hsp68/lacZ enhancer trap vector. Both introns acted as enhancers inducing neuronal expression of the hsp68 minimal promoter in a position- and orientation-independent fashion. As shown in Fig. 5, intron 1 directed hsp68/lacZ expression mainly to the telencephalon, diencephalon, and mesencephalon. The intron 1 enhancer responsible for expression to anterior structure of embryonic nervous system is located in 5′-region of this intron (Fig. 5, construct 5′int-1). The presence of regulatory elements within introns of the human NF-L gene is consistent with the mapping of the DNase I hypersensitive sites (HS) reported by Yazdanbaksh et al. (41). These investigators located three different neuron-specific DNase I hypersensitive sites in the 5′-flanking region (HS1, -2, -3) and four within the human NF-L gene (HS4, -5a, -5b, -6). Except for HS4, located in exon 1, all other intragenic hypersensitive sites are located within introns. The HS5a and 5b are positioned at the 5′-region of intron 1 shown here to contain elements for neuronal expression, while HS6 is located at the end of intron 2.

It is noteworthy that the positions of introns 1 and 2 are conserved among all intermediate filament genes of class IV and VI. It has been postulated that genes of class IV and VI, which correspond to the neuronal branch of the IF family, arose by a reverse transcription of an mRNA from an ancestral lamin-like gene (42). Recently, Zimmerman et al. (43) demonstrated that elements regulating mesodermal and neuronal transcription of the nestin gene, an IF protein of class VI, reside in the first and second introns at positions equivalent to intron 1 and 2 in the NF-L gene. It is thus conceivable that the acquisition of introns by an intronless progenitor gene may have led to the emergence of a lineage of IF genes expressed in the nervous system.

A computer search was carried out to identify known cis-regulatory elements within the promoter and introns of the human NF-L gene. Elements like NF-E1, HOX type 1, and the PEA3 sequences are present in both introns 1 and 2 (Fig. 7). None of these cis regulatory elements can explain neuronal specificity. The mRNA coding for the ETS transcription factor PEA3 is detected in mouse brain, but its expression is not exclusive to this tissue (44). We note, however, that the sequence CAGGA, which includes part of the core consensus AGGAA recognized by the family of ETS transcription factors (45), is present in numerous neuron-specific genes including

**Fig. 5.** Neuron-specific regulatory elements in the NF-L gene direct heterologous lacZ expression. Embryos were analyzed 13.5 days after microinjection of lacZ DNA constructs. At left is shown a schematic representation of each lacZ fusion construct. An × indicates the stained tissues, and the arrow at the end of the line indicates the embryos from which pictures were taken. At right are photographs of embryos from side, back, and front.
the genes for NF-M (8, 46), NF-H, \(\alpha\)-internexin (15), peripherin (47), Drosophila dopa decarboxylase (48, 49), chicken \(\alpha\)7-nicotinic acetylcholine receptor (50), mouse olfactory marker protein (51), rat neuron-specific enolase encoding gene (52), Purkinje-cell-protein-2 encoding gene (53), the gene encoding the growth cone-associated protein SCG10 (54), and type II sodium channel encoding gene (55). Most known members of the ETS family are expressed in lymphoid tissue, but other ETS-related factors are also expressed in neurons (56). We recently reported that an ETS-like element located at \(2908\) in the human NF-L \(5^9\)-region is recognized by a factor expressed in P19 cells differentiated into neurons (56). Moreover, two neuron-specific DNase I hypersensitive sites border this element (41). Our combined results suggest an implication for ETS-related transcription factors in the control of neuron-specific expression. Mutational analysis of the ETS-like elements in the NF-L gene should establish their specific contributions to expression of this gene in the nervous system.

\(^2\) J.-P. Julien, unpublished data.

**Fig. 6.** Histological detection of \(\beta\)-galactosidase in an hNF-L/lacZ transgenic mouse. The brain was dissected from adult F1 transgenic of mouse line 1 (see Fig. 5) after perfusion with 4% paraformaldehyde in PBS. The lacZ staining was performed overnight on 3-mm-thick tissue slices. Frozen sections (15-20 \(\mu\)m) were collected in a cryostat, counter-stained with neutral red, mounted, and pictured. Overview of a near-midline sagittal and transversal brain section is shown (A and B, respectively). Panels showing a higher magnification are as follows: C, the cingulum region (transversal section); D, the lateral septum (transversal section); E, the hippocampus (sagital section); and F, the cerebellum (sagital section). Transversal sections through the spinal cord dorsal horn (G) and the dorsal root ganglia (H) are shown; olfactory nuclei (I) is also shown. Pl, Purkinje cell layer; ML, molecular layer; GL, granule cell layer; V, ventricule; LS, lateral septum; Cg, cingulum; Ep, ependimaries.

**Fig. 7.** Potential regulatory elements in the human NF-L and transcription factor binding sites found in the regulatory region of the human NF-L. The analysis was done on hNF-L regions \(-292\) to +15 (basal promoter), \(-2225\) to +1232 (intron 1), and \(-2450\) to +2834 (intron 2) using the GCC program with the TFD data base from GenBank. No mismatches were allowed with consensus sequences.
Acknowledgments—We thank Catherine Grandjean for technical assistance. The help of Daniel Houle, Gaétan Gagnon, and Debby Altschuller for the production and breeding of transgenic mice is gratefully acknowledged.

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Multiple Neuron-specific Enhancers in the Gene Coding for the Human Neurofilament Light Chain
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J. Biol. Chem. 1995, 270:30604-30610.
doi: 10.1074/jbc.270.51.30604

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