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Structure of the 68-kDa Neurofilament Gene and Regulation of Its Expression*

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The complete structure of the mouse 68-kDa neurofilament (NF-L) gene was elucidated. We cloned cDNAs corresponding to 3.5- and 2.3-kb NF-L mRNA, including their polyadenylation sites. Sequence analysis revealed that these NF-L mRNAs arose from the alternative use of two polyadenylation sites in exon 4. Promoter analysis using NF-L promoter- β -galactosidase fusion plasmids determined regions responsible for its basic promoter activity, which were located between -328 and -36 base pairs from the transcription initiation site. These promoter fusion plasmids induced a significant level of β -galactosidase in NF-nonproducing C6 cells as well as in NF-producing PC12h cells. The *in vitro* transcription assay using HeLa cell extract also showed that this promoter exhibited strong transcriptional activity. Little difference in NF-L mRNA stability was observed between the two cells. However, nuclear run-off assay revealed that the NF-L gene was not transcribed in NF-nonproducing C6 cells. These data suggest that the strong promoter activity of the NF-L gene is repressed *in vivo* at the transcription initiation level in a tissue-specific manner.

In the development of the central nervous system, neuroepithelial cells proliferate and differentiate into neurons and glia. Neurons extend many neurites to form a complex neural network and process information. Glia support neuronal functions in various ways, e.g. making myelin, separating neural cell bodies from each other, and buffering many ions effluxed from excited neurons. Thus their functions are quite different, even though they differentiate from the same precursor cells, neuroepithelial cells. Therefore, unveiling the molecular machinery determining the fate of a neuroepithelial cell is an important initial step in understanding the mechanisms underlying the formation of the complex, yet well organized architecture of the central nervous system. Since the morphological and functional differences between neurons and glia should be caused by the proteins specifically pro-

duced in those cells, we have been studying the mechanisms that govern the expression of neuron-specific or glia-specific genes.

Neurofilaments (NFs)¹ and glial fibrillary acidic protein (GFAP), the proteins specifically produced in neurons and astroglia, respectively, have been used extensively as marker proteins for their identification. Since both of them belong to the intermediate filament family and would seem to be functionally related to each other, we chose to investigate the regulatory system involved in the gene expression of these filaments. In this paper, we focus on studies aimed at understanding the regulation of 68-kDa NF gene expression.

NFs, which are expressed in central and peripheral neurons, are formed from three component proteins with molecular masses of 68 kDa (NF-L), 150 kDa (NF-M), and 200 kDa (NF-H). Recently, many workers have reported the genomic organization of NF proteins (1–11), and the three subunits of NF were shown to be encoded by three different genes. Studies on exon-intron distribution of these genes revealed that NF genes form a class distinct from the rest of the intermediate filament family (2, 4, 6, 7, 11). The NF-L gene is composed of 4 exons, and two species of mRNAs (2.3 and 3.5 kb in size) containing the same coding sequence are transcribed. Although the functional difference in these mRNAs is not clear, the expression pattern of each mRNA is not always coordinately regulated, suggesting that posttranscriptional control contributes to the regulation of the overall level of NF-L transcripts (12, 37).

Here we report the complete structure of the gene encoding mouse NF-L. The variation in size of NF-L transcripts was caused by alternative use of polyadenylation sites. Promoter analysis using the cell lines (NF-producing PC12h and GFAP-producing C6) and the *in vitro* transcription system showed that the NF promoter is a strong promoter *in vitro* and some mechanism which represses this promoter activity *in vivo* may be involved in determining the neuron-specific expression of this gene.

MATERIALS AND METHODS

cDNA Cloning of 3.5-kb Mouse NF-L mRNA—A mouse cerebellum cDNA library in λ gt11 (13, 14) was screened with a ³²P-labeled 3-kb *Eco*RI fragment of a mouse NF-L genomic clone, NF68 Gene (kindly donated by Dr. N. J. Cowan, (2)). Three positive clones, one of which contained poly(dA) tract, were sequenced using double-stranded plasmids as templates (15).

cDNA Cloning of the 2.3-kb NF-L mRNA—The cDNA cloning method using polymerase chain reaction was performed according to Frohman *et al.* (16). Total mouse brain RNA (5 μ g) prepared by the guanidinium/cesium chloride method (17) and a synthetic oligonucleotide primer, T17 adaptor 5'-GGAGCTCTGCAGTCGACAAG-

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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) J05670.

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¹ The abbreviations used are: NF, neurofilament; GFAP, glial fibrillary acidic protein; bp, base pair; kb, kilobase.

C(T)₁₇-3', which contains *Sac*I, *Pst*I, *Sal*I, and *Hind*III recognition sites, were used for cDNA synthesis. NF3' adaptor, 5'-CTCTAGACCTCCTTACGCAGAGTA-3', which contains the *Xba*I recognition site at the 5' end, and T(-) adaptor, 5'-GGAGCTGTCAGTCGACAAGC-3', which is a part of the T17 adaptor, were used for amplification. Synthesized double-stranded cDNA was cloned in the *Hind*III-*Xba*I site of the pUC19 vector after digestion with *Hind*III and *Xba*I.

Cloning of the Promoter Region of NF-L Gene—Mouse chromosomal DNA was digested with *Hind*III and *Xba*I, fractionated on 0.7% agarose gel, and DNA fragments ranging from 2.5 to 3.3/kb in size were collected. They were ligated into the pUC19 vector. This genomic library was screened with a ³²P-labeled probe from NF68 cDNA (1), which was kindly donated by Dr. N. J. Cowan. One of three positive clones named pKNN17'0 was sequenced.

Primer Extension and RNase Mapping—Total mouse brain RNA was prepared according to Maniatis *et al.* (17). Poly(A)⁺ RNA was purified by oligo(dT)-cellulose chromatography (17). The primer extension was done as described previously (18) using a 5'-³²P-labeled synthetic oligonucleotide, 5'-GTCGAAAAGTACGGATC-3', as a primer. RNase mapping was performed as described previously (18). A *Pst*I-*Pvu*II 482-bp fragment of pKNN17'0 was subcloned into pGEM-4 (Promega), and an antisense RNA produced from the SP6 promoter by SP6 polymerase in the presence of [α -³²P]UTP was used as a probe.

Plasmid Constructions—All procedures were done following the standard methods (17). The plasmid containing the mouse genomic NF-L *Hind*III-*Xba*I fragment was digested with *Sma*I and religated in the presence of *Hind*III linker. The resulting plasmid was used as a source for the following construction. To construct pKNN12'0, a 1.7-kb *Hind*III-*Hind*III fragment containing the promoter region was inserted into the *Hind*III site of a promoter-proving vector, pIP110 (38) which contains *Escherichia coli lacZ* gene downstream from the *Hind*III site. The other 5'-truncated mutants were constructed using appropriate restriction sites: *Kpn*I (-933), *Nru*I (-328), *Pst*I (-101), *Msp*I (-36). These sites were changed to *Hind*III sites, and the resulting *Hind*III fragments were inserted into pIP110.

For expression of the NF-L mRNA in C6 cells, we constructed pMTNFg, in which the NF-L promoter of the NF-L genomic clone was replaced by a metallothionein promoter. Initially, the NF mini-gene was constructed using the *Bgl*II site of pKNN17'0 and NF68 cDNA. A *Hind*III-*Bgl*II fragment of pKNN17'0 containing NF-L promoter and a *Hind*III-*Bgl*II fragment of NF68 containing NF cDNA and pUC8 vector sequence were ligated to make pNF-mini. pNF-mini was partially digested with *Sma*I, followed by the *Hind*III linker insertion into one of the *Sma*I sites (+74). This plasmid was digested with *Eco*RI, blunted with Klenow enzyme, and religated in the presence of a *Hind*III linker. The *Hind*III-*Hind*III fragment containing almost the entire NF cDNA was inserted into a *Hind*III site of pMTSV, which was provided by Dr. M. Inouye,² generating pMTSV-NFm. NF68 Gene was digested with *Eco*RI and religated to remove the 3-kb *Eco*RI fragment containing two *Hind*III sites. The resulting pNFg-Δ-E was digested with *Hind*III and *Sma*I (+169) and blunted using Klenow enzyme. Then an *Xho*I (blunted)-*Sma*I fragment from pMTSV-NFm containing the metallothionein promoter and NF-L ATG codon was inserted. Then the 3-kb *Eco*RI fragment of NF68 Gene was inserted back into the original *Eco*RI site to generate pMTNFg. All plasmids used for transfection were purified by two cycles of CsCl density gradient centrifugation.

Cell Culture and Transient Expression Analysis—Rat PC12h cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 5% horse serum. Cells were harvested and 2 × 10⁶ cells suspended in 100 μl of Dulbecco's phosphate-buffered saline were transfected with 20 μg of DNA by the electroporation method using Gene Transfer model GT-11 (M & S Instruments Inc.) at 400 V/cm for 10 ms. Then the cells were plated on 6-cm collagen-coated dishes (Iwaki Glass). Rat C6 cells, maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were transfected with 20 μg of DNA/6-cm dish using the calcium phosphate coprecipitation method with 15% glycerol shock (19). Cells were harvested 48 h after transfection. The β-galactosidase assay was described elsewhere (38).²

In Vitro Transcription—The *in vitro* transcription and S1 nuclease analysis of the transcript were performed as described previously (20). A *Hind*III-*Hind*III fragment from position -328 to +74 of the NF-L gene was subcloned into the *Hind*III site of pBR322. The resulting

plasmid was digested with *Bam*HI and used as a template for *in vitro* transcription. The same fragment (-328 to +74) was 5' end-labeled, and the lower strand was separated and used as a probe for S1 nuclease analysis. Adenovirus major late promoter (-34 to +33) (20), myelin basic protein gene promoter (-1300 to +60) (20), heat shock protein gene promoter (-1200 to +65) (21), and chicken conalbumin gene promoter (-122 to +227) (22) were used as reference promoters for *in vitro* transcription.

Analysis of NF-L mRNA Stability—C6 cells grown in 10-cm dishes were transfected with 20 μg of pMTNFg by the calcium phosphate coprecipitation method. One day after transfection, cells were harvested, mixed together, and replated to avoid differences between dishes. After 48-h incubation in the medium containing 100 μM ZnSO₄, actinomycin D (10 μg/ml) was added. PC12h and the transfected C6 were harvested at various times after actinomycin D treatment. Purification of cytoplasmic RNA was performed according to Greenberg and Ziff (23) and further purified by precipitation with 2 M LiCl. Cytoplasmic RNA was separated on 1.5% formaldehyde agarose gel and transferred to a nylon filter membrane (Zeta-Probe, Bio-Rad). The 1.2-kb *Bgl*II-*Eco*RI fragment of NF68 cDNA and the 2.0-kb *Pst*I fragment of the chicken β-actin cDNA clone, pA1, (24) were labeled by a Random Primer Labeling Kit (Boehringer Mannheim) and used as probes. The filter was washed, dried, and exposed to Kodak XAR-5 film at -70 °C.

Nuclear Run-off Assay—Preparation of nuclei from PC12h and C6 cells and nuclear run-off assay were performed according to Greenberg and Ziff (23) with slight modifications. Run-off product labeled with [α -³²P]UTP was purified using Quick Spin™ columns (Boehringer Mannheim). NF68 cDNA digested with *Eco*RI, pA1 digested with *Pst*I, and pUC18 digested with *Eco*RI were blotted onto a nitrocellulose filter using a slot blot apparatus (Schleicher and Schuell). Ten micrograms of DNA was applied per slot.

RESULTS

Structure of Mouse NF-L Gene—NF-L cDNA as well as its gene have been cloned and their sequences determined (1, 3, 5). The cloned NF-L cDNA corresponded to a smaller 2.3-kb mRNA; however, its polyadenylation site was not shown. Moreover, the structure of a longer 3.5-kb species remained totally unknown. Northern blot analysis of total or poly(A)⁺ mouse brain RNA using various fragments of mouse genomic clone as probes revealed that *fragment II* shown in Fig. 1 specifically hybridized to a 3.5-kb NF-L mRNA (data not shown), suggesting that the two mRNA species arose from alternative use of different polyadenylation sites. In order to address this more directly, three overlapping cDNA clones corresponding to 3.5-kb mRNA were cloned from the oligo-dT-primed λgt11 mouse cDNA library using the 3-kb *Eco*RI fragment (Fig. 1) as a probe. Sequence analysis of these clones led to the conclusion that the 3.5-kb mRNA utilized the

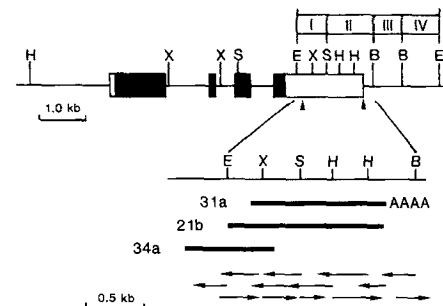


FIG. 1. Schematic representation of mouse NF-L gene. A restriction endonuclease map of the NF-L gene is shown on the top. Coding regions are indicated by filled boxes and noncoding regions by open boxes. Fragments I, II, III, and IV were used as probes to analyze the 3' end structure of the gene (see "Results"). Polyadenylation sites are indicated by arrowheads. An expanded map of the 3' region is shown below. The inserts of λ clones are indicated by thick lines. The poly(A) tract contained in clone 31a is indicated by "AAAA." The regions that were sequenced are indicated by arrows. H, *Hind*III; X, *Xba*I; S, *Sac*I; E, *Eco*RI; B, *Bam*HI.

² S. Inouye and M. Inouye, unpublished results.

polyadenylation site 1.2 kb downstream from the known genomic sequence of exon 4 (Fig. 2B). No additional introns were found.

The "AATAAA" sequence found just upstream of the *EcoRI* site, which formed the most 3' end of the previously isolated NF-L cDNA clone (1), was suggested to be the polyadenylation signal for the 2.3-kb NF-L mRNA. However, the actual polyadenylation site was not determined. For some unknown reason, we could not obtain a clone containing the 3' end of 2.3-kb NF-L cDNA with oligo(dA) tract during the previous experiment. To this end we applied the cDNA cloning strategy with polymerase chain reaction employed by Frohman (16) and successfully cloned cDNAs corresponding to the 3' region of the 2.3-kb NF-L mRNA. T17 adaptor (see "Materials and Methods") was used as a primer for cDNA synthesis from total mouse brain RNA. The resulting cDNA was amplified using the NF3' primer corresponding to the sequence in exon 4 (Fig. 2B) and T(-) adaptor corresponding to the linker region of the T17 adaptor. Amplified double-stranded cDNA was cloned into the pUC19 vector and sequenced. The polyadenylation sites of the 2.3-kb mRNA were positioned 117 and 199 bp downstream from the *EcoRI* site (Fig. 2B). Although two putative polyadenylation signals exist just upstream of the polyadenylation site, it is unclear which of them is critical for polyadenylation. Consequently, the two NF-L mRNA species were synthesized by alternative use of polyadenylation sites.

Next we determined the transcription initiation site by primer extension analysis using the NF primer-2 shown in Fig. 2A (data not shown). The result clearly indicated that 102 (major species) and 106 bp upstream of the ATG codon are the transcription initiation sites of the NF-L gene (see

arrowheads in Fig. 2A). RNase mapping analysis yielded a similar result (data not shown).

The established structure and sequence of the 3' region of the NF-L gene are shown in Figs. 1 and 2B.

Analysis of the NF-L Gene Promoter—To determine the elements of the NF-L gene promoter important for its transcriptional regulation, we performed reporter gene analysis using various NF-L promoter-*lacZ* fusion plasmids. A genomic clone containing a *HindIII*-*XbaI* 2.8-kb fragment of the mouse NF-L gene was also isolated from a recombinant plasmid library with NF68 cDNA probe in our laboratory and served for this purpose. Fig. 3A diagrams the NF-L promoter-*lacZ* fusion plasmids used in this analysis. A rat pheochromocytoma cell line, PC12h, which expresses the NF-L gene, and a rat glioma cell line, C6, which does not express NF-L mRNA, were chosen as recipient cells. The plasmids were transfected by the calcium phosphate method into C6 cell, whereas electroporation was employed for transfection into PC12h because of its low transfection efficiency by the calcium phosphate method. Two days after transfection the cells were harvested and processed for β -galactosidase assay. In both cell lines the plasmid pKNN112'0 containing the 1.6-kb 5'-flanking region were efficiently transcribed. Among a series of deletion mutants tested, a mutant that deleted to position -328 (+1 for transcription initiation site) showed the maximum activity, and further deletion to position -101 decreased promoter activity drastically. Deletion up to position -36 (5 bp upstream of the TATA box) showed no promoter activity. No significant differences were observed in this pattern between NF-L-producing PC12h and nonproducing C6. These data suggest that at least two *cis*-acting promoter elements for efficient transcription, regardless of the tissue-restricted

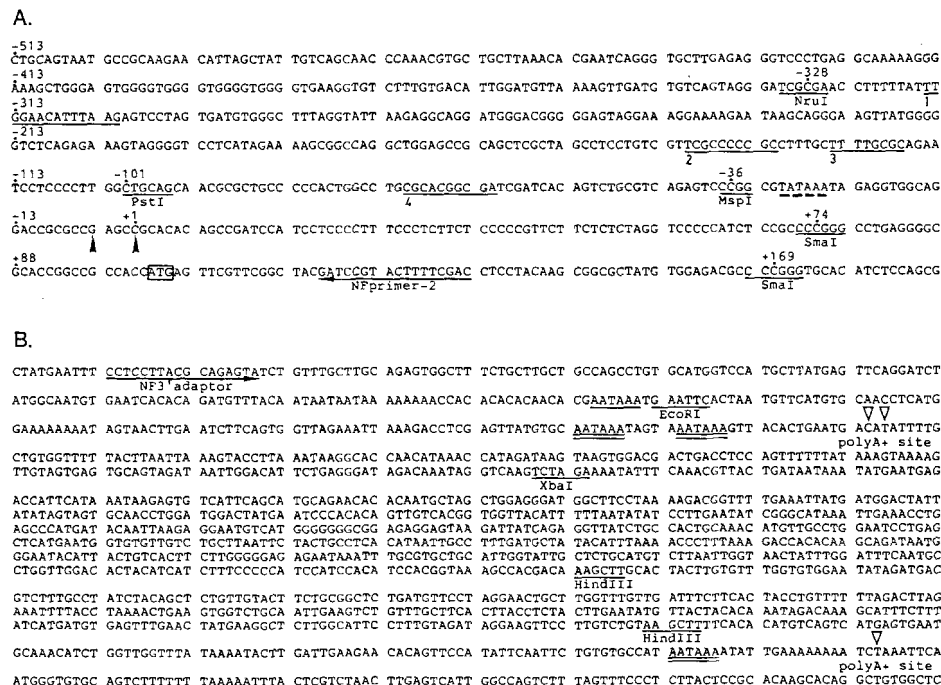


FIG. 2. Nucleotide sequence of the mouse NF-L gene promoter region and 3' region. A, the sequence of the NF-L gene promoter region is shown. The transcription initiation sites determined by primer extension analysis (NF primer-2) and RNase mapping are shown by arrows. The translation initiation codon (ATG) is surrounded by a box and the TATA-like sequence indicated by a dotted line. Numbers indicate the relative position from the major transcription initiation site. The underlines with numbers indicate the sequences which have homology with known elements: 1, heat shock transcription factor; 2 and 4, AP-2 binding site; 3, E2F binding site. B, the sequence of the 3' region of the NF-L gene is shown. The primer used in cDNA cloning (NF3' adaptor, see "Materials and Methods") is indicated by an arrow. The polyadenylation sites are indicated by open triangles and their possible polyadenylation signals by double lines.

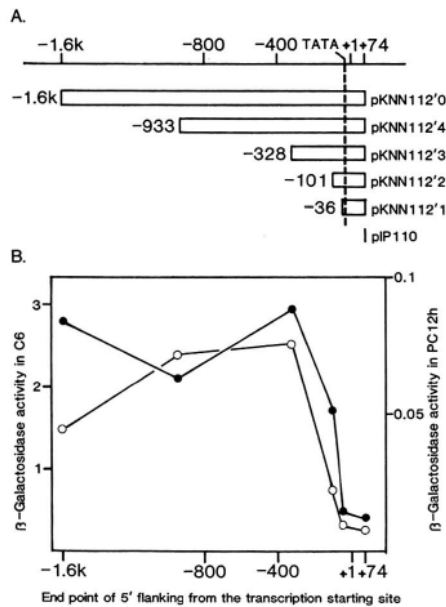


FIG. 3. Deletion analysis of the mouse NF-L gene promoter region. A, the 5'-truncated mutants of the NF-L gene promoter region are shown. Numbers indicate the relative position as shown in Fig. 2. These promoter regions were cloned into the pIP110 vector, which contains the *E. coli lacZ* gene. B, β -galactosidase activity of the cells transfected with the plasmids are plotted. The scale for the activity in C6 (●) is indicated on the left side and that for PC12h (○) on the right. Unit of β -galactosidase activity is defined by nanomoles of *o*-nitrophenyl- β -D-galactopyranoside cleaved per min/mg of protein. -1.6k represents -1600.

expression, seemed to be present in the regions from -328 to -101 and from -101 to -36. We found sequences which have homology with known elements in this region. The sequence homologous to the AP-2 binding site T/CCC/GCCA/CN(GCG)/(CGC) (25, 26), is located at -141 and at -71 in opposite orientation. The sequence homologous to the heat shock transcription factor binding site CTNGAANNTTC-NAG (27, 28) is located at -315, matching 8 bases out of 10 nucleotides. The sequence homologous to the E2F binding site TTTCGCGC (29, 30) is located at -125, matching 7 out of 8 nucleotides. These elements recognized by a ubiquitous activator might contribute to the fundamental promoter activity.

Detection of elements responsible for tissue-specific promoter activity by comparing transient promoter activities in different cell lines is difficult, because it is necessary to normalize differences in transfection efficiency or other parameters. We calculated the transfection efficiency of C6 cells and PC12h cells by counting the number of β -galactosidase positive cells using fluorescein-di- β -D-galactopyranoside as a substrate (38). The transfection efficiencies of C6 cells and PC12h cells were 7.1 and 4.8%, respectively. This difference in transfection efficiency could not explain the higher NF-L promoter activity in C6 cells compared with PC12h cells, because the β -galactosidase activity in C6 cells was about 40 times higher than that in PC12h cells. These data suggested that either tissue-specific activating elements were not present in the DNA fragment used in this experiment or were not functional in the transient assay system.

To provide direct evidence that the promoter activity of the NF-L gene was not tissue-restricted, at least in the promoter region we tested, *in vitro* transcriptional assay using HeLa cell extract was performed. The fragment containing position -328 to +74, which showed maximum promoter activity in the transient promoter assay, was used as a template. The

result (Fig. 4A) showed that the NF-L promoter was more efficiently transcribed than the promoters (myelin basic protein, adenovirus major late, chicken conalbumin, HSP70) employed as controls. The fact that the NF-L promoter is more active in the *in vitro* transcription system than the adenovirus major late promoter, which is considered to be a strong promoter, indicates that the NF-L promoter itself is functional in various cell lines if general transcription factors, those found in HeLa cells, are provided. The transcripts from the NF-L promoter fragment used in this assay were initiated from the correct CAP sites, as shown by the S1 nuclease protection assay (Fig. 4B). Therefore, repression of this efficiently transcribable gene must have taken place in NF-L nonproducing cells.

Expression of Endogenous NF-L mRNA Is Regulated at the Transcriptional Level in PC12h and C6—Recently, we have shown that the level of NF-L mRNA could be regulated posttranscriptionally in PC12h-R cells (37). It is possible that the expression of NF-L mRNA is regulated by rapid degradation of the transcribed mRNA in non-neuronal cells. To test this hypothesis, we examined the degradation rate of NF-L mRNA in PC12h and C6. Since no transcripts from the endogenous NF-L gene were detectable in C6 cells, we used DNA transfection to force expression of the NF-L gene to measure its degradation rate in C6. We constructed a plasmid pMTNFg in which metallothionein promoter was substituted for the NF-L promoter by replacing the 1.7-kb *HindIII*-*SmaI* fragment of the NF-L genomic clone with a 0.7-kb *XhoI*-*HindIII* fragment containing the metallothionein promoter. The C6 cells transfected with pMTNFg transiently expressed two NF-L mRNAs that had the same size as endogenous rat NF-L mRNA expressed in PC12h on Northern blot analysis. PC12h without transfection and the C6 cells transfected with pMTNFg were harvested at appropriate times after treatment with actinomycin D. Cytoplasmic RNA was purified and subjected to Northern blot analysis for NF-L mRNA. The results (Fig. 5) indicated that no major differences in degradation rate were observed between PC12h and C6. It even seemed that the degradation of the NF-L mRNAs, particularly that of the 2.3-kb species, was much more rapid in PC12h

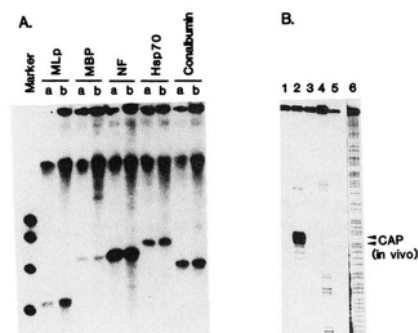


FIG. 4. *In vitro* transcription of NF-L gene in HeLa cell extracts. A, run-off transcription from the NF-L promoter and other promoters. Marker used was pBR322 digested with *MspI*. Lanes of MLp, MBP, NF, HSP70, and conalbumin show the run-off transcripts from adenovirus major late promoter, mouse myelin basic protein gene promoter, NF-L promoter, heat shock protein gene promoter, and chicken conalbumin gene promoter, respectively. The template DNAs used in reactions (lanes a, 0.225 μ g; lanes b, 0.45 μ g) had been linearized by appropriate restriction endonuclease. Signals of expected run-off products are observed in the lower half of the film. B, analysis of the transcription initiation site of *in vitro* transcripts by S1 mapping. Lanes: 1, yeast RNA; 2, *in vitro* transcript; 3, *in vitro* transcript with α -amanitin; 4, probe; 5, G + A reaction of the probe; 6, longer exposure of lane 5. The transcription initiation sites *in vivo* are indicated by arrowheads.

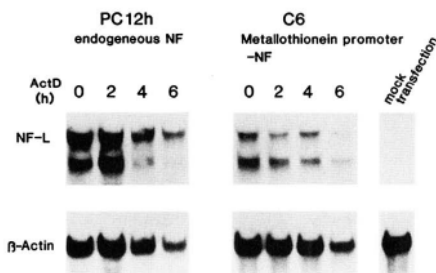


FIG. 5. Effect of RNA synthesis inhibitor on the NF-L mRNA in PC12h and C6 cells. Northern blot analysis of NF-L mRNA transcribed from the endogenous gene in PC12h cells and from exogenous metallothionein promoter NF-L gene in C6 cells was performed after actinomycin D (*ActD*) treatment (10 μ g/ml). Twenty μ g of cytoplasmic RNA prepared at the indicated times after actinomycin D treatment was fractionated by formaldehyde agarose gel electrophoresis, blotted to nylon filters, and hybridized with a 32 P-labeled NF-L cDNA probe or β -actin cDNA probe. The filters were exposed to an x-ray film at -70°C . In the lane of mock transfection, cytoplasmic RNA from C6 cells transfected with no DNA was used.

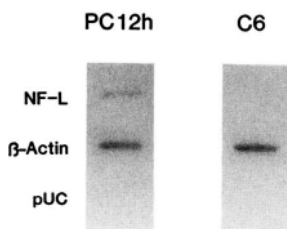


FIG. 6. Analysis of the NF-L gene transcription in PC12h and C6 cells. NF-L cDNA, β -actin cDNA and pUC19 vector were spotted onto nitrocellulose filters and hybridized with 32 P-labeled run-off transcripts from nuclei isolated from PC12h and C6 cells. The filters were autoradiographed at -70°C in the presence of intensifying screen.

than C6. Hence, it is unlikely that the NF-L mRNAs were stabilized in a tissue-restricted manner in neuronal cells after they had been transcribed without tissue specificity. Therefore, posttranscriptional regulation does not seem to take place in C6 cells in order to shut down NF-L gene expression.

To confirm that the regulation of NF-L gene expression truly occurred at the transcriptional level, we measured the rate of transcription by nuclear run-off assay. Fig. 6 shows that the NF-L gene transcripts were undetectable in NF-L nonproducing C6, whereas the β -actin gene was transcribed at nearly equal amounts in PC12h and C6. Thus, it is clear that the machinery conferring neuron-specific expression on the NF-L gene is acting at the transcription initiation level, although its promoter region alone may be functional in various cells.

DISCUSSION

We have determined the 3' ends of the NF-L gene and established its complete structure. The two NF-L mRNAs arose from alternative use of different polyadenylation sites (Fig. 2B). The transcription initiation sites we determined were in nearly the same position described by Monteiro and Cleveland (31). To investigate the molecular mechanisms which regulate expression of neuron-specific genes, we have studied the NF-L gene promoter and the stability of its transcripts. Although NF-L is normally expressed in neurons *in vivo*, the NF-L promoter was quite active in both neuronal and non-neuronal cell lines in transient promoter assay (Fig.

3B). It was also efficiently transcribed in the *in vitro* transcription system using HeLa cell extracts (Fig. 4A). Since we found that NF-L mRNAs were stabilized in PC12h-R cells in response to NGF (37), we investigated the possibility of posttranscriptional regulation in determining the neuron-specific expression of the NF-L gene. However, we could not observe C6-specific destabilization of NF-L mRNAs, excluding the above possibility. Previous reports showed that when the whole NF-L gene was transfected into non-neuronal cells, NF-L mRNA was transiently expressed (human NF-L gene into L cells (4), mouse NF-L gene into L cells (31)). Our data using NF-L promoter-*lacZ* fusion plasmids and metallothionein promoter-NF-L gene fusion were consistent with these observations. However, the human NF-L gene (32) and the mouse NF-L promoter/HSV-1 transactivator VP16 chimeric gene (33) were expressed in a tissue-specific manner in transgenic mice. In the latter case, especially, the promoter region used was the same as the region we examined in this study ($-1.6\text{ kb HindIII}/+74\text{ SmaI}$). Although they employed an indirect detection system (VP16 driven by NF promoter activates a reporter gene for detection), the neural tissue-specific distribution of reporter gene expression (except for slight expression in heart) indicates that the NF-L promoter region contains elements necessary for neuron-specific expression. These findings together with our results from the nuclear run-off assay suggest that the tissue-specific activity of the NF-L promoter depends on the state of the DNA containing the NF gene. It seems that the NF-L promoter was repressed in non-neuronal cells when exogenous DNA was integrated in the chromosome. It must be determined whether the exogenous NF-L promoter is repressed in non-neuronal cells after integration into the chromosome. However, our preliminary data using stable transformant cells suggested that integration of the NF-L promoter region into the chromosomal DNA is not sufficient for tissue-specific expression.³ The NF-L promoter-*lacZ* fusion gene was expressed in stable transformants of a fibroblast cell line, whereas GFAP promoter and promoterless control DNA exhibited no promoter activity. Modification during ontogenesis such as change in methylation pattern probably is necessary for tissue-specific regulation. Although this could not exclude the presence of neuron-specific activator(s) which play a critical role in the neuron-specific expression of the NF-L gene, at least there must be some mechanisms which prevent NF-L gene expression in non-neuronal cells. In order to identify elements involved in neuron-specific regulation, it is important to use DNA with a configuration similar to that of chromosomal DNA.

We observed that 2.3-kb mRNA degraded faster than the 3.5-kb species in PC12h, similar to the result in PC12h-R cells, whereas the rate of degradation was nearly equal in C6 cells (Fig. 5). The 2.3-kb mRNA in PC12h cells degraded even faster than that in C6 cells. Moreover, the level of 2.3-kb mRNA was higher than that of the 3.5-kb species in C6 cells, whereas the reverse was observed in PC12h cells. This suggests that 2.3-kb NF-L mRNA is specifically destabilized in PC12h cells. Because the difference between 2.3- and 3.5-kb mRNA is that the 3.5-kb species has an additional 3'-noncoding region, the elements for destabilization should be present near the 3' end of 2.3-kb mRNA. We searched for AUUUA repeats, which are known to destabilize mRNA (34), but were unable to find this sequence in NF-L mRNA. Although the reason for mRNA destabilization in NF-L expressing cells is not known, it is possible that it is involved in preventing the

³ I. Fujimoto, K. Ikenaka, K. Nakahira, and K. Mikoshiba, unpublished observation.

overproduction of NF protein, which may disrupt the cell structure.

GFAP, an intermediate filament the same as NF, is expressed in astroglial cells. Because of their close relation, such as conserved protein structure, we considered that we could identify common regulatory elements and/or neuron/glia-specific elements by comparing the regulatory mechanisms of their gene expression. However, the results showed that the mechanism regulating the NF-L gene was quite distinct from that for the GFAP gene. We found that the GFAP promoter exhibits its specificity in a transient expression system and that astrocyte-specific *cis* elements are present with 256 bp of the 5'-flanking region.⁴ The NF genes belonging to type IV intermediate filaments have a different gene structure from those of GFAP, vimentin and desmin, which belong to type III intermediate filaments (35, 36). Studies on exon-intron organization suggest that NF genes might have diverged before other intermediate filament genes evolved (4) or might have originated through a reverse transcription event from mature mRNA of an ancestral intermediate filament gene (2, 7). This difference in evolutionary pathway may explain the different regulatory mechanisms.

We have shown that multiple mechanisms were involved in the regulation of NF-L gene expression. First, the neuron-specific expression of the NF-L gene was primarily controlled at the transcription initiation level. Perhaps some chromosomal structure which represses expression in non-neuronal cells is involved in the mechanism. Second, the levels of NF-L transcripts seemed to be mainly controlled by its promoter activity. We showed that *cis* elements were present in the region between -328 and -36. Whether ubiquitous or neuron-specific factors act on these elements in neurons remains to be determined. Finally, the specific destabilization mechanism suggests that the levels of NF-L transcripts were also controlled posttranscriptionally. It is of interest that one of the two species of NF-L mRNA was regulated. This neuron-specific posttranscriptional control would contribute to the fine regulation of the level of the mRNA. Further analysis on these mechanisms could reveal the regulation system of the neuronal marker gene.

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Structure of the 68-kDa neurofilament gene and regulation of its expression.

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