High levels of neurofilament (NF) mRNA expression are attained during early postnatal development and are a major determinant of axonal size. High level NF expression is also dependent upon axonal continuity since NF mRNA levels are down-regulated after nerve transection. This study shows that both postnatal up-regulation and axotomy-induced down-regulation are altered by deletion of 3'-UTR from the mouse light NF subunit (NF-L). Transgenes with (NF-L-I) or without (NF-L-I) 3'-UTR display similar patterns of neuron-specific expression but differ in their respective levels of expression. Whereas changes in the level of NF-L mRNA parallel those of the endogenous mouse NF-L mRNA, changes in the level of NF-L mRNA differ from the pattern of endogenous NF-L expression during postnatal up-regulation and axotomy-induced down-regulation. Specifically, the NF-L transgene undergoes a 3-fold aberrant up-regulation between embryonic days 15 (E15) and 18 (E18) and has lost its susceptibility to axotomy-induced down-regulation. Studies of transfected P19 cells show that 3'-UTR deletion leads to a several-fold stabilization of NF-L mRNA and an increase in steady-state mRNA level. The findings support the working hypothesis that the 3'-UTR contains determinants that alter stability and that stabilization of NF-L mRNA regulates the levels of NF-L mRNA in neuronal tissues and cells.

Large axons are composed predominantly of neurofilaments (NFs) so that their rate and size of impulse conduction are dependent on the expression, assembly, and transport of the component light (NF-L), mid-sized (NF-M) and heavy (NF-H) NF subunits. Expression, assembly, and transport of the endogenous NF gene products are normally coordinated so that NFs do not accumulate, and axonal size is determined by the levels of NF subunit expression within the cell (1, 2). Expression of exogenous NF-L (3), NF-H (4), or mutant NF-H (5) transgenes can be disruptive to NF assembly or transport and lead to proximal NF accumulations and to reduced NF content and size of distal axons. Reduced expression of endogenous NF-L also leads to NF accumulations and reduction in axonal size as well as diminished expression of NF-M and NF-H (6). Axonal size may also be influenced by NF phosphorylation or by interactions with Schwann cells (7, 8).

NF expression arises in post-mitotic neurons (9) but remains at low levels throughout embryonic development (10). High level NF expression takes place during early postnatal development (11), coincident with widespread enlargement and myelination of axons (12). The postnatal up-regulation is manifested by coordinate increase in NF mRNA and proteins during time frames that reflect regional differences in neuronal maturation (11). In primary sensory neurons, the postnatal surge of NF expression correlates closely with increases of NF content and with enlargements of perikaryal size and axonal caliber (13).

Studies in our laboratory have shown that the postnatal up-regulation of NF expression is accompanied by increases in stabilities of NF-L, NF-M, and NF-H mRNAs in primary cultures of rat sensory neurons (14). The same NF transcripts in adult sensory neurons are destabilized in vitro in a transcription-dependent manner (15) by a process that resembles the coordinated down-regulation of NF mRNAs following axotomy (16). These findings have led to the view that NF mRNAs are stabilized in high-expressing neurons and that changes in mRNA stabilities may account for the coordinate up-regulation and down-regulation of NF mRNA levels that occur during postnatal development and in axotomized neurons, respectively. Moreover, the conservation of 3'-UTR in NF mRNAs (14) and the involvement of 3'-UTR in mRNA stability (17, 18) suggest that the 3'-UTR in NF genes may harbor determinants of NF expression.

The present study has begun to test the above hypothesis in vivo by comparing expression of the endogenous mouse NF-L gene with that of a marked mouse NF-L transgenes in which the 3'-UTR is intact (NF-L-I) or deleted (NF-L-I). Our findings identify the 3'-UTR of the mouse NF-L gene as instrumental in mediating both the developmental up-regulation and the axotomy-induced down-regulation of NF-L. The findings indicate the importance of 3'-UTR in NF-L expression and highlight the potential role of posttranscriptional mechanisms in regulation of neuron-specific gene products.

**MATERIALS AND METHODS**

Construction of a Marked NF-L Transgene—The mouse NF-L gene (19-21) was marked by inserting a 66-bp fragment (ATCCGCTT-GAGGCGCAAGGCGCGAGCGCTATCGCCCTAGGGCCAAG-GCCGCAAGGCCCTG) into the BglII site (+829) of exon 1 (where +1 is the start site of transcription). The in-frame addition included SfiI, NarI, and StuI restriction sites and ablated the BglII site. The insert was made from complementary oligonucleotides with protruding BglII ends. Orientation was determined by sequencing. Oligonucleotides were synthesized on an Applied Biosystems model 391 DNA synthesizer.
Construction of Marked NF-L Genes with Intact (NF-L*) or Deleted (NF-L-3' UTR–A PCR fragment from +3312 (PFMI) to +4100 (with NotI site added) of NF-L was cloned into the PFMI/NotI sites in the 3'-end of the marked NF-L gene to yield NF-L*. The sequence extended 74 bp beyond the polyadenylation signal (+4026) that forms the 2.5-kilobase NF-L mRNA. A 3'-UTR deletion was created by two-step PCR (22). Reaction A used an upstream primer containing the PFMI site (+3312) and a downstream antisense primer (number 3) that juxtaposed the 5' (+3614) and 3' (+3985) end points of the 3'-UTR deletion. Reaction B used a downstream primer with a NotI site (+4100) and an upstream sense primer (number 4) that was complementary to primer 3. The products of reactions A and B were used with the upstream (+3312) and downstream (+4100) primers in a third round of PCR to create a fragment with PFMI/NotI ends but lacking 3'-UTR sequence from +3615 to +3984. This fragment was cloned into the NF-L marked gene to yield NF-L**.

Generation of Transgenic Mice—Transgenic mice were obtained by microinjecting male pronuclei of fertilized mouse eggs with marked NF-L genes that had been linearized by Arai/NotI digestion, fractionated by agarose gel electrophoresis, electroeluted, ethanol-precipitated, and passed through an Elutip ion exchange column (Schleicher and Schuell). Microinjections were conducted by the University of Pennsylvania Transgenic Mouse Core Facility on eggs harvested from superovulated B6SJLF1/J females mated with B6SJLF1/J males. Transgenic founder mice were identified by PCR analysis of genomic DNA using sense (363/376) and antisense (892/915) oligonucleotide primers that spanned the marked insert. Copy numbers were determined by a Molecular Dynamics PhosphorImager analysis of Southern blots using genomic DNA that had been cut with PvuI/StuI to liberate a 720-bp fragment from the endogenous NF-L gene (1391/1101) and a 448-bp fragment from the marked NF-L transgene (+382/+829). Blots were probed with a PvuI/BglII (+391/+827) fragment that had been labeled with random primers to a specific activity of 10^9 dpm/μg. Founder mice were expanded by mating with B6SJ F1J mice.

Transfection of Cells—Neuro2A, P19, and L cell lines, obtained from the American Type Culture Collection (Rockville, MD), were cotransfected with the marked NF-L* and NF-L- genes and a plasmid, pRc/CMV (Invitrogen), containing the Escherichia coli neo gene. Stable transfectants were selected by growth in G418. Cells were transfected with DNA that was coprecipitated with calcium phosphate (21) or treated with Lipofectin (Life Technologies, Inc.). Amounts of DNA and methods of transfection were optimized for each cell line. Stable transfectants were pooled (from over 500 colonies) to reduce disparities in transgene copy number.

Northern blots of stably transfected L cells (which only express the transgenes) show that the NF-L* transgenes generate a single mRNA species that comigrates with the 2.5-kilobase NF-L mRNA isoform of mouse spinal cord, while the NF-L* transgene produces a single mRNA species of about 2.1 kilobases (data not shown).

The NF-L transgenes also contained an insert at the BglII site (+829) that enables transcripts from the transgenes and endogenous mouse NF-L gene to be distinguished by RNase protection assay (see Fig. 1). With an antisense probe that spans the insertion site, fragments of 190 and 110 nucleotides are generated from the transgene and endogenous mouse NF-L genes, respectively. Protection assays show that NF-L* and NF-L- mRNAs are readily expressed in stably transfected L cells and are coexpressed with endogenous mouse NF-L mRNA in P19 cells (Fig. 2). The NF-L* and NF-L- transgenes generate similar steady-state mRNA levels in L cells but not in P19 cell lines. Levels of NF-L* mRNA are higher than NF-L- mRNA in stably transformed P19 cells even though the lines were derived from pooled transfectants and contained similar average copy numbers of the respective transgenes (5–10 copies/pool). The presence of NF-L* or NF-L- increased levels of endogenous NF-L (Fig. 2), whereas the presence of
NF-L also increased levels of NF-M and NF-H mRNA (data not shown).

Expression of NF-L+ and NF-L− Transgenes in Transgenic Mice—NF-L+ and NF-L− sequence (−325/+4100) were excised from the parent plasmids, purified, and used to create transgenic mice. Introduction of NF-L+ into the mouse germ line produced four founder mice (of 22 pups) and established three transgenic lines (A, B, and C). Separate microinjections of the NF-L− yielded three (of 42 pups), two (of 27 pups), and one (of 17 pups) founder mice. Three lines (D, E, and F) of NF-L− were established. All lines (A–F) contained two to four copies of integrated transgenes.

Expression of the transgene versus endogenous NF-L genes was analyzed in neural and non-neural tissues of F1 adult mice from each cell line by RNase protection assay. Transgene expression was detected in every line, most readily in brain and spinal cord, but levels of expression did not correlate with transgene copy number. Steady state levels of NF-L− mRNA were, on average, at least 2-fold higher than that of NF-L+ mRNA. Ectopic expression occurred in non-neural tissue but at levels that were at least 10-fold lower than that of brain and spinal cord. Minute amounts of transgene mRNA were detected in thymus, kidney, lung, testis, spleen, and liver.

Lines A and D expressed the highest levels of NF-L+ and NF-L− transgenes and were used to assess the effects of 3′-UTR deletion on the regional expression of the transgenes in the nervous system. Both NF-L+ and NF-L− transgenes showed similar rank order patterns of expression relative to...
the expression of the endogenous NF-L gene (Fig. 3). Increased transgene/endogenous mRNA expression was also noted in brain versus spinal cord tissues of other transgenic lines (data not shown). The findings indicate that the NF-L<sup>1</sup> and NF-L<sup>2</sup> transgenes are expressed in a similar distribution, albeit at differing levels, in the nervous system. Although the expression of the transgenes differed from that of the endogenous NF-L gene, the differences were not due to deletion of the 3'-UTR. Interestingly, the pattern of NF-L transgene expression is consistent with that of other neuronal transgenes that have been examined at the cellular level (see Ref. 24), namely partial (or mosaic) expression in neurons that express the endogenous gene and ectopic expression in neurons that do not express the endogenous gene.

Developmental Expression of NF-L<sup>1</sup> and NF-L<sup>2</sup> Transgenes—Transgene and endogenous NF-L mRNA levels were analyzed in brain and spinal cord at embryonic days 15 (E15) and 18 (E18) and at postnatal days 2 (P2), 10 (P10), and 20 (P20) in the F2 generation of lines A (NF-L<sup>1</sup>) and in lines D and F (NF-L<sup>2</sup>). Endogenous NF-L mRNA, present in E11 mouse brain (25), is readily detected in brain and spinal cord of transgenic mice at E15 and remains at about the same level at E18 and P2 (Figs. 4 and 5). NF-L<sup>1</sup> mRNA is also detected in brain and spinal cord at E15 and remains at roughly the same level at E18 and P2. NF-L<sup>2</sup> mRNA is also present at E15 but undergoes a 2-fold increase by E18 and a 3-fold increase by P2 in both brain and spinal cord. Similar up-regulations of NF-L<sup>2</sup> mRNA in brain and spinal cord at E18 and P2 were also observed in brain and spinal cord in a second line (F) of transgenic mice (data not shown). NF-L<sup>2</sup> and NF-L<sup>1</sup> mRNAs were
also expressed at very low levels in liver at E15, but no consistent developmental up-regulation of ectopic liver expression was detected (data not shown). Comparative analyses of mRNA levels at P2, P10, and P20 indicate that endogenous NF-L mRNA increases in spinal cord and brain at P10 and at P20 and that NF-L and NF-L mRNAs are also up-regulated at these time points. The extent of postnatal up-regulation at P10 and P20 varied among individual mice but, on average, was similar for endogenous NF-L and for the NF-L transgenes in brain and in spinal cord.

Expression of NF-L and NF-L Transgenes in Axotomized Neurons—The effects of sciatic nerve transections on NF mRNA levels in sensory neurons of the L4/L5 DRG were examined in F1 and F2 transgenic mice at 6–10 weeks of age. Preliminary studies on non-transgenic littermates showed that the down-regulation of NF-L, NF-M, and NF-H mRNAs in axotomized sensory neurons (15) is reproduced in the mouse strain (26–31). Deletion of 3'-UTR in NF-L does not change the pattern of tissue-specific expression but alters the extent of gene expression during developmental up-regulation and axotomy-induced down-regulation of NF-L. In addition, deletion of 3'-UTR is associated with increased steady-state mRNA levels in transgenic mice and in stably transfected P19 cells.

Alterations in the developmental expression of the 3'-UTR-deleted transgene (NF-L*) occur during the transition from low level to high level NF gene expression. This transition normally occurs during early postnatal development and is part of a coordinated postnatal up-regulation of all three NF genes (11). Deletion of the 3'-UTR leads to an aberrant up-regulation of the NF-L transgene at E18 during late embryonic development. The aberrant up-regulation of the NF-L* transgene at E18 does not alter the expression of the endogenous NF-L gene or prevent the subsequent postnatal up-regulation of the mutant transgene. Hence, a direct relationship between the aberrant embryonic and postnatal up-regulations is not readily apparent.

Deletion of NF-L 3'-UTR also prevents the down-regulation that occurs in DRG neurons following sciatic nerve transection. This axotomy-induced loss of NF mRNA levels in high expressing adult DRG is related to postnatal NF up-regulation in the sense that it does not occur in axotomized DRG neurons if the transections are made prior to the period of postnatal up-regulation (32). Hence, both postnatal up-regulation and axotomy-induced down-regulation are associated with high level expression.

The phenotype of the mutant transgene (NF-L*) is complex in that it results in a gain of function (premature up-regulation) during development and a loss of function (sensitivity to axotomy) in mature neurons. A gain of function in a deletion mutant implies that the 3'-UTR contains repressive elements during development, while the loss of function suggests that factors that lower NF-L expression can no longer function in mature neurons. Such diverse effects in a deletion mutant suggest that positive and negative elements are deleted or that the cognate binding factors are inoperative in the respective target neurons. The changes could reflect alterations at transcriptional or posttranscriptional levels of function. The latter is favored by the complexity of the phenotype and its limitation to quantitative, not distributional, features of expression.

Moreover, the ability of the 3'-UTR deletions to increase the...
half-life of NF-L transcripts in P19 cells provides direct evidence that increased NF-L expression can result from stabilization of NF-L transcripts and that differential stabilization is manifest by mRNAs derived from NF-L+/NF-L− transgenes. Additional studies in our laboratory indicate that NF-L− mRNA is also more stable than NF-L+ mRNA in brain polyribosome preparations from transgenic mice. Although limited by variable nonspecific degradation, this in vitro model indicates that factors regulating mRNA stability of the NF-L−/NF-L+ transgenes are present in primary neurons as well as in P19 cells.

While our findings support the view that the NF-L 3′-UTR is instrumental in regulating mRNA stability, the regulatory elements and underlying mechanisms are unknown. For example, it is unclear whether the aberrant up-regulation (E18) reflects a differential maturation of stabilizing or destabilizing factors that can selectively affect the NF-L− or NF-L+ transgenes. It is also possible that the aberrant embryonic (E18) and postnatal up-regulations may involve common regulatory components that destabilize (or stabilize) NF-L mRNA and become functional at different times during development. Deletion of 3′-UTR may also alter the balance of stabilizing versus destabilizing components and lead to increased steady-state levels of NF-L mRNA in cell lines and transgenic tissues that contain active cognate binding factors.

Involvement in mRNA stability may partly account for the conservation of 3′-UTR sequence in the NF (14) and other genes (33). However, transcript stabilization/destabilization often involves sequences within the coding region or multiple cis-acting elements and may be closely associated with translation (for reviews, see Refs. 17, 18, and 35–37). Hence, 3′-UTR deletions may alter only a part of the stabilizing or destabilizing pathway. It is also likely that stabilization of NF-L mRNA serves to coordinate levels of NF-L mRNA with those of NF-M and NF-H mRNAs and with the levels of NF metabolism in the cells. The present study identifies the 3′-UTR as a key regulatory region in NF-L expression and provides a framework for identifying additional components that regulate NF mRNA levels within the cell.

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