Stability Determinants Are Localized to the 3’-Untranslated Region and 3’-Coding Region of the Neurofilament Light Subunit mRNA Using a Tetracycline-inducible Promoter*

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The tetracycline-responsive expression system of Bujard was used to compare rates of decay of wild-type and mutant neurofilament (NF) light subunit (NF-L) mRNAs. Optimal conditions for activation and inactivation of the target transgene were determined using a luciferase reporter gene. Analyses of mRNA stability were therefore carried out on cells that were doubly transfected with transactivator and inducible target genes and derived from pooled clones of transfected cells. Rates of mRNA decay were compared upon inactivation of the transgenes after high levels of mRNA had been induced. Deletion of the 445-nucleotide (nt) 3’-untranslated region (3’-UTR) (L/+/+++) or 527 nt of the 3’-coding region (3’-CR) (L/++++) increased the stability of NF-L mRNA compared with the full-length (L/+++++) transcript in neuronal (N2a and P19 cells) and non-neuronal (L cells) lines. Deletion of both the 3’-UTR and 3’-CR (L/++--) led to a further stabilization of the transcript. A major stability determinant was then localized to a 68-nt sequence that forms the junction between the 3’-CR and 3’-UTR of NF-L and is the binding site of a unique ribonucleoprotein complex (Cañete-Soler, R., Schwartz, M. L., Hua, Y., and Schlaepfer, W. W. (1998) J. Biol. Chem. 273, 12655–12661). The studies establish a novel system for mapping determinants of mRNA stability and have applied the system to localize determinants that regulate the stability of the NF-L mRNA.

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Altering mRNA stability is a universal post-transcriptional mechanism for modulating gene expression during growth, development, and differentiation. Post-transcriptional regulation may be particularly important in cells, such as neurons, that undergo extensive differentiation and change after cessation of cell division. Alterations in mRNA stability are known to affect the levels of several key proteins in post-mitotic neurons (2–5). Increases in mRNA stability accompany the postnatal up-regulation in neurofilament (NF) expression and are, therefore, instrumental in determining the size and shape of neuronal processes (6, 7). Moreover, levels of NF expression have been implicated in motoneuron disease by virtue of the selective motoneuron degeneration that occurs when a light (NF-L) or heavy (NF-H) subunit transgene is overexpressed in transgenic mice (8, 9). A transient up-regulation of endogenous NF mRNA also precedes the spontaneous motoneuron degeneration in the Wobbler mice (10).

Functional assays for mapping stability determinants use a variety of methods to compare stabilities of mutant versus wild-type mRNAs (11). Most of these methods are limited by their disruptive or pleiotrophic effects on the host cells and are, therefore, especially problematic for assessing stabilities of long-lived mRNAs. An alternative method is provided by the development of the tetracycline-responsive promoter system whereby target gene expression is stringently controlled by a non-toxic antibiotic ligand (12–19). Here, we have adapted this system to assess mRNA stability in neuronal and non-neuronal cell lines and to map stability determinants in the NF-L transcript. The method has enabled the identification of stability determinants in the 3’-CR and 3’-UTR of the NF-L mRNA and have localized a key determinant to a 68-nt sequence at the junction of the 3’-CR and 3’-UTR.

EXPERIMENTAL PROCEDURES

Plasmids—pUHD15.1, pUHD10.3, and pUHD13.3 were a generous gift of Dr. H. Bujard. pUHD15.1 is a modified expression vector that was rendered autoinducible (see Ref. 16) by placing an EcoRI site and consensus translation start sequence immediately upstream of the tTA open reading frame and subcloning the EcoRI/BamHI PCR fragment from pUHD1.1 into the polylinker of pUHD10.3.

Construction of Wild and Mutant NF-L cDNAs—Full-length and mutant mouse NF-L cDNAs (see Fig. 1) were constructed by splicing together three PCR fragments. Fragment 1 extended from +1 to a BglII site at +829 in exon 1, fragment 2 extended from +829 to a KpnI site at +1208, and fragment 3 extended from +1208 to a site (+2180) 25 bp upstream of the AATAAA termination signal. The PCR template for fragment 1 was a genomic clone of NF-L, and templates for fragments 2 and 3 were cDNAs generated from mouse spinal cord. HindIII and XbaI sites were placed in the flanking primers to facilitate cloning of cDNAs into the HindIII/XbaI polylinker sites of pRC/RSV (Invitrogen, San Diego, CA) and pRC/tet (modified pRC expression vector in which the Rous sarcoma virus promoter was replaced with the heptameric Tn-10 tet operator sequence). The pRC/tet vector containing the full-length NF-L cDNA was designated L/++. A deletion mutant lacking 445 bp of 3’-UTR (L/++++) was created by splicing PCR fragment 3, which extended 3′ to a site (+1740) just beyond the TGA stop codon. A deletion mutant lacking 445 bp of 3’-UTR and 527 bp of the coding region (L/++++) was generated by splicing PCR fragments 1 and 2 and placing a TGA stop codon at +1212. A deletion mutant lacking only the 3’-coding region (L/++++) was made by splicing with PCR fragment 3 containing a KpnI site, TGA stop codon, and EcoRI site, and 3’-UTR. A 68-bp deletion at the junction of 3’-CR and 3’-UTR (+1755) was generated by splicing upstream and downstream PCR fragments that lacked sequence between +1712 (BspMI site) and +1797 (HincII site) and placing a stop codon at +1712. Wild-type sequence in L/++++ was then replaced with the PCR fragment and designated L/+/+deB++. Constructs were...
sequenced to confirm the integrity of junctional and Tn-10 tet operator sequences.

**Cell Lines/Transfections**—HeLa, P19, N2a, and 3T3 (L) cell lines were obtained from the American Type Culture Collection (Rockville, MD) and transiently or stably transfected using calcium phosphate (20) or LipofectAMINE (7). Cells were cotransfected with transactivator (pUHD15.1 or pUHD15.1M) and pSVZeoc (Invitrogen), selected with Zeocin, and pooled (>100 clones). Cells were then transfected with target genes bearing tetracycline-responsive promoters, selected with G418 and Zeocin, and pooled (>100 clones). Growth and selection were conducted in the presence of tetracycline (0.5 μg/ml). The presence of the transgenes was monitored by PCR.

**Assay of Tetracycline-responsive Reporter Gene**—The luciferase reporter gene driven by the tetracycline-responsive promoter (pUHD13.3) was used to test tetracycline-mediated regulation of transcription. Pooled clones of N2a, P19, and L cells bearing pUHD15.1 or pUHD15.1M were transiently transfected with pUHD13.3 and assayed for luciferase activity after 24 h in the absence of tetracycline. pUHD13.3 was also stably transfected by cotransfected with pcDNA3 (Invitrogen). Luciferase activity was measured by luminometer (Lumat LB 9501; Berthold, Wildbad, Germany) using a luciferase kit (Promega, Madison, WI) according to manufacturer’s instructions.

NF-L expression was compared in pooled clones of N2a, P19, and L cells that had been doubly transfected with pUHD15.1M and pRChet vectors bearing wild-type or mutant NF-L cDNAs. Cells were grown in the absence of tetracycline, split into replicate plates, and harvested in triplicate for NF expression at varying time points after readdition of tetracycline. Ribonuclease protection assays were conducted as described previously (3).

**RESULTS**

**Characterization of Transactivator (tTA) Function on P19, N2a, and L Cells**—A luciferase reporter gene with tetracycline-responsive promoter (pUHD 13.3) was used to test the ability of the tTA transactivator expression vectors (pUHD15.1 or pUHD15.1M) to confer tetracycline-sensitive activation in neuronal (P19 and N2a) versus non-neuronal (L cells) cell lines. Preliminary studies with transient transfections revealed increases in luciferase activity of 2–4 orders of magnitude in neuronal and non-neuronal cell lines by 24 h after withdrawal of tetracycline (data not shown), as reported previously in other cell lines (12–14). A similar range of induction was observed in cell lines that had been transiently transfected or that had been stably transfected with the tTA transactivator vector. Expression vectors with constitutive (pUHD15.1) and autoinducible (pUHD15.1M) promoters were both effective in conferring tetracycline-responsive activation.

To monitor the tetracycline-responsive promoter system for both activation and inactivation of the test gene, the luciferase reporter gene was stably transfected into cell lines containing the autoinducible tTA transactivator expression vector (pUHD15.1M). To reduce position effects and disparity in copy numbers, multiple clones were pooled for each transfection. The luciferase gene in doubly transfected cell lines was highly sensitive to tetracycline (Fig. 2A) and was rapidly activated and inactivated upon withdrawal and readdition of tetracycline (Fig. 2B and C). Induction factors ranged from 100- to 300-fold after 24 h and reached maximal levels by 72 h after withdrawal of tetracycline in the neuronal and non-neuronal cell lines. There was also rapid decreases of luciferase activity upon readdition of tetracycline, with less than 5% and 0.5% of residual activities after 24 and 48 h, respectively. Levels of tetracycline-inducible luciferase expression did not differ appreciably in cell lines transfected with varying admixtures of tTA expression vectors driven by constitutive (pUHD15.1) and autoinducible (pUHD15.1M) promoters.

**Tetracycline-induced Transactivation of NF-L Expression**—NF-L cDNAs driven by the tetracycline-responsive promoter were readily inducible upon withdrawal of tetracycline in P19 and N2a cells (Fig. 3) as well as in L cells (data not shown). Increases of 50–100-fold in NF-L mRNA levels occurred in P19 and N2a cell lines in which the tTA expression (pUHD15.1M) and NF-L target (L(++++) vectors) had been transfected. NF-L expression was not induced in the parental N2a cells lacking a NF-L transgene (Fig. 3, parental); however, there was low level leakage from the L(++++) transgene in the presence of tetracycline (Fig. 3, 0 h). Background in P19 cells was also derived from endogenous NF-L gene expression. Interestingly, marked increases of NF-L transgenic expression in P19 cells was accompanied by up-regulation of endogenous NF-M gene expression. Occasionally, there also appeared to be slight increases in NF-H expression.

**Tetracycline-induced Inactivation of Wild-type Versus Mutant NF-L Transgenes Demonstrates Stability Determinants in the 3'-CR and 3'-UTR of the NF-L mRNA**—The stabilities of wild-type versus mutant NF-L mRNAs were compared by inducing NF-L transgene expression for 72 h and then measuring mRNA levels at varying time points after the readdition of tetracycline. Fig. 4 depicts a representative experiment of mRNA decay in P19 cells containing wild-type (L(++++)) or mutant (L(++++), L(+++), and L(++-)) NF-L target transgene. Fig. 5 shows the average decline of NF-L/β-actin mRNA levels in N2a and P19 cells from three experiments. Similar pattern of NF-L mRNA decay was observed in L cells (data not shown). In all instances, wild-type (L(++++)) NF-L mRNA was stably transfected into cell lines containing the autoinducible tTA transactivator expression vector (pUHD15.1M). To reduce position effects and disparity in copy numbers, multiple clones were pooled for each transfection. The luciferase gene in doubly transfected cell lines was highly sensitive to tetracycline (Fig. 2A) and was rapidly activated and inactivated upon withdrawal and readdition of tetracycline (Fig. 2B and C). Induction factors ranged from 100- to 300-fold after 24 h and reached maximal levels by 72 h after withdrawal of tetracycline in the neuronal and non-neuronal cell lines. There was also rapid decreases of luciferase activity upon readdition of tetracycline, with less than 5% and 0.5% of residual activities after 24 and 48 h, respectively. Levels of tetracycline-inducible luciferase expression did not differ appreciably in cell lines transfected with varying admixtures of tTA expression vectors driven by constitutive (pUHD15.1) and autoinducible (pUHD15.1M) promoters.

**Stability Determinants in NF-L mRNA**
relatively unstable, comparable to measurements using actinomycin-induced decay (3). Deletion of 3’-CR (L/+/-) or 3’-UTR (L/+/-) diminished the loss of NF-L mRNA in each of

the cell lines tested. Moreover, deletion of both 3’-CR and 3’-UTR (L/+/+) was additive in that it further diminished the loss of the NF-L transcript. Short term (6 h) losses of NF-L mRNAs from wild-type and mutant transgenes in P19 cells showed intermediate levels of decline (data not shown), supporting the view that decreases in the rate of loss of NF-L/β-actin mRNA over time may be due to increasing admixtures of mRNA from the endogenous NF-L gene. The loss of NF-L mRNA upon inactivation of the transgene in P19 cells was accompanied by a parallel loss of NF-M mRNA expression that had been up-regulated by induction of the transgene. Interestingly, the up-regulation (and subsequent down-regulation) of the endogenous NF-M gene did not occur with high level expression of the L/+/+ mutant NF-L mRNA.

Localization of Stability Determinant to the Distal 23 nt of 3’-Coding Region and Proximal 45 nt of 3’-UTR of the NF-L Transcript—To localize stability determinants in NF-L transcript, a NF-L transgene (L/+/del/-) was constructed with a 68-nt segment deleted from the junction between the coding region and 3’-UTR. The deleted sequence corresponded to the binding site of the C-binding RNP complex (1) and comprised the 23 nt of distal 3’-CR and 45 nt of proximal 3’-UTR. Loss of NF-L mRNA from the deleted transgene (L/+/del/-) was much less than that from the wild-type transgene (L/+/+). Deletion of either the 3’-CR (L/+) or 3’-UTR (L/+) led to intermediate levels of stabilization. A diminished rate of mRNA decay from the deleted transgene (L/+del/) was also observed in L cells (data not shown). The findings indicate that a major stability determinant is localized to the junction between the 3’-CR and 3’-UTR.
DISCUSSION

The tetracycline-responsive promoter system provides for stringent, non-toxic, and high level control of specific gene expression using either the addition or withdrawal of the antibiotic ligand (or derivatives) to activate or repress transcription (12–19). The present study has adapted this system to study mRNA stability using the ligand to turn off transcription after high levels of the test gene mRNA are attained. For this purpose, the use of tetracycline as a repressor is advantageous in that the kinetics of the off reaction is mediated by the addition rather than by the disappearance (i.e., half-life) of the ligand. Our methods have also incorporated some safeguards such as the pooling of stably transfected clones to equalize test gene copy number, reduce position effects, and limit alterations from genetic perturbations during passage of the transgenes. Pooling of transfectants may reduce levels of induction but generates sufficient and comparable levels of transgene

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**FIG. 4.** Protection assay of NF-H (H), NF-M (M), NF-L (L), and β-actin (βA) mRNA levels in P19 cells in which transcription of wild-type (L/++++) and mutant (L/+−−−, L/++−−, and L/++++−) NF-L transgenes was activated for 72 h in the absence of tetracycline and then inactivated by addition of tetracycline (0 time point).

**FIG. 5.** NF-L mRNA decay in P19 and in N2a cells following activation and inactivation of transcription of wild-type (L/++++) and mutant (L/++−−, L/++−−, and L/++++−) NF-L cDNAs, as described in Fig. 4. NF-L mRNA levels were quantitated by phosphorimager and normalized to levels of β-actin mRNA. Data were averaged from three experiments.

**FIG. 6.** A, protection assay of NF-L (L) and β-actin (βA) mRNAs in N2a cells transfected with mutant (L++/del/++) or wild-type (L/++++) transgene after tetracycline-induced inactivation of the transgenes. B, loss of NF-L/β-actin mRNAs in N2a cells with mutant (L+/del/++) or wild-type (L/++++) transgene. Data were quantitated by phosphorimager and averaged from three experiments.

**TABLE I**

| Transgenes     | Half-lives (h) |
|----------------|----------------|
| L/++++         | 17.2           |
| L/++−−         | 22.5           |
| L/++++−        | 34.0           |
| L/+−−−/del/++  | 48.2           |
mRNAs for ready assessment of transcript decay.

The relative persistence in steady-state levels of mutant versus wild-type NF-L mRNA after inactivation of the transcript indicates that deletion of either the 3′-coding region (L/+++++) or the 3′-UTR (L/++++−) removes destabilizing components from the NF-L transcript. The increased stability of the double mutant (L/++++−) mRNA suggests that sequences in the 3′-CR and in the 3′-UTR may also act in an additive or cooperative manner. Multiple determinants in the 3′-coding region and 3′-UTR have also been detected in other neuronal transcripts (21, 22) and could function in separate pathways (see Refs. 23 and 24) or as separate components of a common pathway. The latter possibility is supported by the localization of a major stability determinant to a 68-nt sequence at the junction of 3′-CR and 3′-UTR of NF-L. Both the 3′-CR and 3′-UTR sequence in the 68-nt fragment are necessary for assembly of a unique C-binding RNP complex at this site (1). Interestingly, the stability of the α-globin mRNA is also regulated by a determinant on the proximal edge of the 3′-UTR that is dependent upon sequence in the adjacent 3′-CR (25, 26).

The increased stability of the mutant NF-L transcripts in both neuronal and non-neuronal cells suggests that destabilization of NF-L mRNA involves common components that are widely present in different cell lines. Moreover, the relative instability of wild-type NF-L mRNA in both neuronal and non-neuronal cell lines, compared with the extraordinary stability of the transcripts in primary neurons (3), raises the possibility that NF mRNA stability is regulated by altering a default destabilization pathway. Such putative stabilizing conditions (or factors) might help to explain the discrepancy between the high levels of NF expression that are attained in vivo and the low levels of NF expression that occur in vitro. It also indicates that destabilization of NF mRNA may provide key insights into the mechanisms regulating levels of NF expression and their role in motoneuron degeneration.

The increases in steady-state levels of endogenous NF-M mRNA that accompany high level expression of the wild-type (L/+++++) or single mutant (L/++++−) transgenes in P19 cells could be due to the titering of a component(s) that serves to destabilize the NF-L and NF-M transcripts. The instability of the double mutant (L/++++−) transgene to up-regulate NF-M expression may indicate that the function of the putative component(s) in destabilizing NF-L and NF-M mRNAs requires both the 3′-CR and 3′-UTR. Similar changes were not observed in N2a or L cells but would have been precluded by methylation and nonexpression of the NF-L and NF-M genes in these cell lines (27). Up-regulation of NF-L upon overexpression of an NF-M transgene has been reported in transgenic mice (28), although the relationship between transgene and endogenous gene expressions may not be comparable to that in P19 cells by virtue of the greater stability of NF mRNA in vivo as well as the much higher levels of transgene expression achieved in vitro.

Our biochemical studies have not only identified the binding site of a unique C-binding RNP complex at the junction of 3′-CR and 3′-UTR but have also detected multiple binding sites of a U/A-binding RNP complex in the 3′-CR and 3′-UTR of NF-L mRNA (1). Moreover, binding of the C-binding and U/A-binding complexes show a pattern of reciprocal interactions, as if competing for common factors or binding sites. To what extent the different complexes enhance or alter the stability of NF-L transcript is presently unknown. The tetracycline-responsive promoter system, herein described, provides a functional assay that will enable a more precise mapping of regulatory elements and, thereby, facilitate the identification and cloning of cognate binding factors that regulate neurofilament gene expression.