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Mutation in Neurofilament Transgene Implicates RNA Processing in the Pathogenesis of Neurodegenerative Disease

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A mouse neurofilament light subunit (NF-L) transgene with a 36 bp *c-myc* insert at the end of the coding region was found to have neuropathic effects on enteric and motor neurons of transgenic mice. The severity of phenotype was related directly to the levels of transgenic mRNA expression. High levels of transgene expression were lethal to newborn pups, causing profound alterations in the development of the enteric nervous system and extensive vacuolar changes in motor neurons. Lower levels of transgene expression led to a transient stunting of growth and focal alterations of enteric and motor neurons. Because the positioning of the *c-myc* insert coincided with the location of the major stability determinant of the NF-L mRNA (Cañete-Soler et al., 1998a,b), additional studies were undertaken. These studies showed that the *c-myc* insert alters the ribonucleoprotein (RNP) complexes that bind to the stability determinant and disrupts their ability to regulate the stability of the transcripts. The findings indicate that expression of an NF-L transgene with a mutant mRNA stability determinant is highly disruptive to enteric and motor neurons and implicate alterations in RNA processing in the pathogenesis of a neurodegenerative condition.

Key words: neurofilament transgene; motor neuron degeneration; transgenic mice; post-transcriptional regulation; ribonucleoprotein complexes; mRNA stability

The degeneration of motor neurons in mice bearing a neurofilament (NF) transgene indicates a selective susceptibility of motor neurons to an unknown component of NF transgene expression. An unknown aspect of NF expression also affects the course of motor neuron degeneration in mice bearing a mutant superoxide dismutase-1 (SOD-1) transgene (Couillard-Despres et al., 1998; Williamson et al., 1998). The neuropathic effects of a NF transgene on motor neurons were detected initially on overexpression of a mouse light (NF-L) or human heavy (NF-H) NF transgene (Cote et al., 1993; Xu et al., 1993), but a much more severe form of motor neuron degeneration occurred in mice bearing a mutant NF-L transgene with a leucine-to-proline point mutation in the rod domain of the protein (Lee et al., 1994). The latter mutation was intended to disassemble NFs, yet expression of the mutant protein did not lead to a granular disintegration of NF profiles, as characteristic of a dominant filament-disassembling subunit (Gill et al., 1990), nor prevent the accumulation of assembled NFs, admixed with mutant protein, in cell bodies and dystrophic neurites of degenerating motor neurons. It is, therefore, unclear whether the accumulations of NFs are primary or secondary events of motor neuron degeneration or why the accumulations should occur so selectively in motor neurons. The accumulation of NFs in degenerating motor neurons has led to the view that the disruptive effects of NF transgenes are attributable to altered NF transport (Collard et al., 1995; Bruijn and Cleveland, 1996), although disruption of NF transport recently has been discounted in the pathogenesis of other neurodegenerative disorders (Eyer et al., 1998).

An alternative view as to the nature of motor neuron degeneration in mice expressing a NF-L transgene has arisen from studies on NF mRNA stability (Cañete-Soler et al., 1998a,b). These studies indicate that the mutant NF-L transgene that causes massive motor neuron degeneration (Lee et al., 1994) also contained a second mutation by virtue of a 36 bp *c-myc* tag that was inserted inadvertently into the major stability determinant of the transcript. The *c-myc* insert separates components in the coding region and the 3′-UTR of NF-L mRNA that are essential for the binding of ribonucleoprotein (RNP) complexes to the stability determinant (Cañete-Soler et al., 1998b). The findings raise the possibility that motor neuron degeneration may be attributable to expression of mutant mRNA rather than mutant protein by the NF-L transgene. Expression of the mutant NF-L mRNA could alter the RNP components that regulate NF-L expression as well as expression of other gene products, such as those that override apoptosis and maintain neuronal viability (see Easton et al., 1997).

The possibility that the *c-myc* mutation in the NF-L transgene is responsible for the neurodegenerative effects would have important implications for the pathogenesis of the neurodegenerative state. Specifically, it would imply that the neuropathic effects are attributable to expression of NF mRNA rather than NF protein by the transgene. This conclusion is supported by studies showing that appending the same *c-myc* tag to the C terminus of the NF-L, NF-M, or NF-H subunit does not affect that ability of the subunit to assemble into filaments (Gill et al., 1990; Wong and Cleveland, 1990; Lee et al., 1993). Moreover, expression of the *c-myc* tag in a NF-M transgene is incorporated readily into assembled NFs without altering neuronal viability in transgenic

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mice (Wong et al., 1995a). Furthermore, because the aforementioned NF-M transgene also contained the 3′-UTR from the mouse NF-L (Wong et al., 1995a), the neuropathic effects of the *c-myc* mutation in the NF-L transgene could be attributed only to the context of the *c-myc* mutation in relation to the stability determinant of the NF-L transcript (Cañete-Soler et al., 1998b). Finally, the possibility that the neuropathic effects of the mutant NF-L transgene could be explained by the stabilization of the message and the overexpression of the protein must be discounted because the severe neuropathic effects from the mutant NF-L transgene occur at reduced levels of NF-L protein expression (Lee et al., 1994; Bruijn and Cleveland, 1996), whereas the neuropathic effects of the wild-type NF-L transgene were observed only when there was a fourfold increase in the expression of NF-L protein (Xu et al., 1993).

The present study has begun to test our working hypothesis by examining the biological effects of a NF-L transgene with only the *c-myc* mutation, the functional effects of the *c-myc* mutation on NF-L mRNA stability, and the biochemical effects of a *c-myc* mutation on the RNP complexes that bind to the stability determinant. We show that expression of the NF-L transgene with the *c-myc* mutation has profound disruptive effects on neurons in the peripheral nervous system and that the *c-myc* mutation alters the binding of RNP complexes and their ability to regulate the stability of the NF-L mRNA. The findings implicate alterations in RNA processing in the pathogenesis of a neurodegenerative state and provide novel insights into the nature of neurodegenerative disease.

MATERIALS AND METHODS

Construction of mutant NF-L cDNAs. A full-length mouse NF-L cDNA (Cañete-Soler et al., 1998a) in the *Hin*dIII/*Xba*I polylinker sites of pSK$^+$ (Stratagene, La Jolla, CA) was used as a PCR template to insert a 36 bp *c-myc* insert immediately upstream of the stop codon. Overlapping sense and antisense primers to the *c-myc* insert (upper case), stop codon (underlined), and NF-L sequence (lower case) were synthesized as follows: CTCATTTCTGAAGAGGACTTGATT<u>tga</u>gccctattcccaactattcc (sense) andTTCAGAAATGAGCTTTTGCTCCATatctttcttcttagccacc (antisense). PCR fragments of upstream (1.7 kb) and downstream (0.5 kb) NF-L sequence were generated by using primers to pSK$^+$ vector sequence flanking the *Hin*dIII and *Xba*I restriction sites. A full-length NF-L cDNA with 36 bp *c-myc* insert then was generated by PCR, using the same flanking primers and the 1.7 kb upstream and the 0.5 kb downstream NF-L PCR fragments as template. The 2.2 kb PCR fragment was gel-excised, cut with *Hin*dIII and *Xba*I, and ligated into the *Hin*dIII/*Xba*I sites of a pRC/CMV expression vector (Invitrogen, San Diego, CA). The integrity of the NF-L cDNA, *c-myc* insert, and stop codon was confirmed by sequencing both strands of the final construct.

NF-L cDNAs were constructed with the same *c-myc* insert inserted into the *Bgl*II site (+828) of exon I (NF-L/*c-myc*/*Bgl*II) or into the *Eco*RI (+2055) in the distal 3′-UTR (NF-L/*c-myc*/*Eco*RI). In each instance, sense and antisense oligonucleotides containing the 36 bp *c-myc* sequence flanked by *Bgl*II or *Eco*RI restriction sites were synthesized, annealed, cut, and ligated into the respective restriction sites of the NF-L cDNA and sequenced to determine the orientation of the *c-myc* insert. Insertion of the *c-myc* sequence into the *Blg*II site did not alter the open reading frame of the cDNA. The integrity of all constructs was confirmed by sequencing.

The NF-L/wt and NF-L/*c-myc* cDNAs were converted into templates for RNA probes by PCR, using primers that bracketed the 23 bp of 3′-coding region (3′-CR) and 45 bp of 3′-UTR and with the T7 promoter sequence appended to the sense primer. The same strategy was used to construct a control *c-myc* probe with the 23 bp of upstream and 45 bp of downstream sequence that flanked the *c-myc* sequence of the *Bgl*II site (+828) in exon I, using the NF-L/*c-myc*/*Bgl*II cDNA as template.

Determination of mRNA stability. cDNAs with wild-type sequence (NF-L/wt), with the stability determinant deleted (NF-L/del), and with *c-myc* mutations (NF-L/*c-myc*, NF-L/*c-myc*/*Blg*II, and NF-L/*c-myc*/*Eco*RI) were placed into the *Hin*dIII/*Xba*I polylinker sites of a pRC/RSV vector (Invitrogen) in which the RSV promoter had been replaced with the heptamerized Tn-10 tet operator sequence, as previously described (Cañete-Soler et al., 1998a). The modified vectors (NF-L/wt/tet, NF-L/del/tet, and NF-L/*c-myc*/tet) were transfected into Neuro 2a cells containing a tTA transactivator cDNA with an autoinducible promoter (Cañete-Soler et al., 1998a). Cells with transactivator and inducible target transgenes were selected by growth in Zeomycin and Neomycin, respectively, and the presence of the transgenes was monitored by PCR. Multiple clones (>100) with both transgenes were pooled. mRNA was assayed by ribonuclease protection assay, and levels of NF-L mRNA were normalized to those of β-actin mRNA in transfected cells. Radioactivity was quantitated by phosphoimager.

Transient transfections were conducted to compare the effects of the *c-myc* insert when placed in the stability determinant (NF-L/*c-myc*/tet), in exon 1 (NF-L/*c-myc*/*Bgl*II/tet), or in the 3′-UTR (NF-L/*c-myc*/*Eco*RI/tet). These vectors were cotransfected with equal amounts of wild-type vector (NF-L/tet) in Neuro 2a cells containing the tTA transactivator expression vector. Expression of the target NF-L cDNAs was activated for 12 hr by growth in the absence of tetracycline, and NF-L mRNAs were quantitated at 24 and 48 hr after the addition of tetracycline.

NF-L mRNAs in transient transfected cells were quantitated by RT-PCR. RNA was extracted from a Qiagen column (Hilden, Germany) and used as a template for reverse transcriptase with random hexanucleotides as primers. 20-mer PCR primers were chosen that extended the PCR products across the sites of the *c-myc* inserts, i.e., from +790 to +925 in exon 1 of NF-L (for NF-L/*c-myc*/*Bgl*II), from +1701 to +1836 (for NF-L/*c-myc*), and from +2000 to +2135 (for NF-L/*c-myc*/*Eco*RI). Antisense primers were admixed at a 1:50 ratio with ^{32}P end-labeled primers. Samples were run for 15, 20, and 25 cycles and separated on 5% acrylamide gels; radioactivity in the PCR products from the mutant and wild-type transcripts was quantitated by phosphoimager.

Gel-shift and cross-linking of RNP components. Full-length RNA probes were labeled uniformly with ^{32}P-UTP, eluted from acrylamide gels, and diluted to 2.5×10^4 cpm/μl immediately before use, as previously described (Cañete-Soler et al., 1998b). Gel-shift and cross-linking assays were conducted with 5×10^4 cpm of probe and 160 μg of protein extracted from rat brain cytosol containing (in mM) 50 K-acetate, 3 Mg-acetate, 2 dithiothreitol, and 20 HEPES buffer, pH 7.4, with or without homoribopolymer competitors. RNP complexes on RNA probes were cross-linked by 30 min exposures on ice at 3 cm under a UV light (4×10^6 J/cm^2), and the radioactive polypeptides were denatured by boiling in SDS sample buffer and fractionated by SDS-PAGE. High-speed cytosolic extracts were obtained from rat brain and were used fresh or within a 4 month period of storage at −80°C. Radioactivity in gel-shifted and cross-linked bands was quantitated by phosphoimager.

Transgenic mice. The NF-L cDNA with 36 bp *c-myc* insert and hCMV promoter was excised with *Xho*I and *Xba*I and microinjected into fertilized eggs of B6SJF1/J female mice that had been mated with B6SJF1/J males. Genomic DNA was extracted from tails of 14 d pups and used to detect the transgene by PCR and to estimate transgene copy number by Southern blot. PCR primers spanned the sequence between +1708 and +1815 and generated PCR fragments of 108 and 144 bp from the wild-type and mutant sequence. Genomic DNA was cut with *Sac*I (+1350) and *Hin*cII (+1814) to generate fragments of 464 and 500 bp from the endogenous NF-L gene and NF-L transgene, respectively. These fragments were separated on a 2% agarose gel and hybridized with radioactive cDNA probes made by random primed labeling of the *Sac*I/*Hin*cII fragment.

Tissue analyses. Transgenic and nontransgenic littermates were euthanized with CO_2 and their brains were excised for RNA protection assay; vertebral columns, abdominal contents, and hindlimb musculature were dissected to expose the tissue for optimal fixation. The intestines were fixed *in situ* by immersion in 10% neutral buffered formalin (NBF) for 24 hr at 4°C and washed and stored in PBS; representative sections were dehydrated and embedded in paraffin. Microscopic sections were stained with hematoxylin and eosin (H&E) or immunostained with PGP9.5 to delineate enteric neurons (Karaosmanoglu et al., 1996). Antibodies to PGP9.5 (Biogenesis, Sandown, NH) were applied at a 1:1000 dilution for 1 hr at room temperature and visualized with goat anti-rabbit biotinylated antiserum and the avidin/biotin detection system (Vector Laboratories, Burlingame, CA). Then the chromophore was developed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO).

After 4 hr of fixation, the vertebral columns of 14 and 28 d and adult mice were dissected further to expose the spinal cords directly to NBF before the washing and storing of tissues in PBS. The spinal cords were

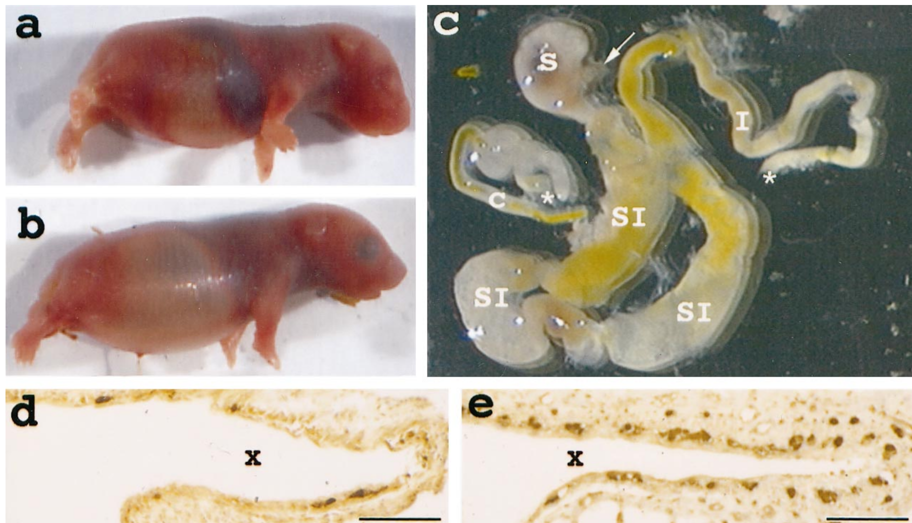

Figure 1. Newborn founder mice A (*a*) and B (*b*) bearing the NF-L transgene with *c-myc* mutation have markedly distended abdomens. *c*, The intestine of pup A reveals a dilated and shortened small intestine (*SI*) between the stomach (*S*) and ileum (*I*). The distal end of the esophagus (*arrow*) and discontinuous ends of ileum (*) and colon (*C*) are identified also. Immunohistochemistry for PGP9.5 (Karaosmanoglu et al., 1996) delineates brown reactive products in the enteric ganglia in the walls of small intestine from pup A (*d*) and a nontransgenic littermate (*e*) that are everted and folded back on themselves and around the abdominal cavity (*x*). The wall of the control is studded with immunoreactive enteric ganglia in the myenteric plexus immediately below the serosal surface. In contrast, there is a marked reduction of immunoreactive enteric ganglia in the wall of the small intestine of pup A. Scale bars: *d, e*, 100 μm.

separated from the vertebral columns, dehydrated, and embedded in paraffin. Paraffin-embedded spinal cords then were cut and positioned in paraffin blocks to obtain serial microscopic cross sections from the cervical to the lumbar cord. Spinal cords were stained with hematoxylin or H&E or were immunostained with primary antibodies to NF-L (N5139; Sigma), to the phosphorylated epitopes on NF-H and NF-M (Ta51; Carden et al., 1987; Lee et al., 1987), and to the human *c-myc* tag (AB1; Calbiochem, La Jolla, CA). Secondary antibodies were biotinylated anti-rabbit or anti-mouse IgGs.

Expression of transgenic and endogenous NF-L mRNA. Brains from transgenic and nontransgenic littermates were homogenized in 4 M guanidinium thiocyanate, and total RNA was extracted and stored at −80°C in formamide (Chomczynski and Sacchi, 1987). Levels of mRNA from the endogenous NF-L gene and from the mutant NF-L transgene were quantitated by RNA protection assay, using radioactive antisense RNA probes that spanned the 36 bp *c-myc* insert (+1770). Templates for the RNA probes were generated by PCR and spanned NF-L sequence from +1525 to +1846, including the 36 bp *c-myc* insert and T7 promoter sequence that was appended to the antisense primer. A full-length RNA probe was labeled uniformly with ^{32}P-UTP by T7 polymerase (Schwartz et al., 1992, 1995), separated by electrophoresis, excised from acrylamide gels, eluted overnight into 0.5 M NH_4 acetate, 0.1% SDS, and 1 mM EDTA, and precipitated with ethanol (Cañete-Soler et al., 1998b). An RNA protection assay was undertaken by hybridizing brain RNA (10–20 μg) with the RNA probe (10^4 cpm), as previously described (Schwartz et al., 1992). Protected fragments of 212 and 322 bp from the wild-type and mutant mRNA were separated by electrophoresis on 7.5% denaturing acrylamide gels, and radioactivity of the protected fragments was detected by autoradiogram and quantitated by phosphoimager.

RESULTS

Expression of NF-L transgene with a 36 bp *c-myc* tag insert leads to a loss of enteric neurons and malformation of the small intestine

To test whether the *c-myc* mutation in an NF-L transgene might have disruptive effects in transgenic mice, we constructed a mouse NF-L transgene with the 36 bp *c-myc* tag at the end of the coding region but without the leucine-to-proline mutation in the rod domain of the protein (see Lee et al., 1994). The transgene was placed behind a strong constitutive promoter and microinjected into the mouse germ line. Nine founder mice (of 67 pups) were recovered. Two founder mice (pups A and B) were born in an agonal state with markedly distended abdomens (Fig. 1*a,b*). Examination of the intestines revealed extensive dilatation of the midgut in pup A (Fig. 1*c*) and, to a lesser extent, in pup B. No specific sites of intestinal perforation were identified. Milk products were not present, but fecal content was observed throughout the intestines, indicating that the intestinal dilatation was not attributable to complete obstruction of the alimentary canal.

Microscopic examination showed that intestinal dilatation was associated with a marked depletion of neurons from the enteric nervous system when the population of enteric neurons was visualized by their immunoreactivity to PGP9.5 (Karaosmanoglu et al., 1996). Multiple sections of midgut revealed either an absence or paucity of neurons in the dilated and thinned intestinal walls of the transgenic pups (Fig. 1*d*) as compared with nontransgenic littermate controls (Fig. 1*e*). The aganglionic and hypoganglionic (loss of >50%) segments of midgut differed only in the extent of neuronal loss. Residual neurons were observed only in the myenteric plexus, although neurons of the myenteric and submucosal plexi were seen in controls. Residual enteric neurons did not display any distinctive pathological features and were difficult to identify with certainty on H&E-stained sections.

Transgene expression leads to vacuolar degeneration of motor neurons and alterations of muscle development

The alterations in the enteric nervous system raised questions as to whether the transgene also might affect the development of other neurons, specifically motor neurons. Microscopic examinations at multiple levels of spinal cord revealed a vacuolar degeneration of anterior horn cells in the transgenic pups (Fig. 2*a,b*). The perikarya of altered motor neurons were filled with vacuoles of variable sizes, irregular shapes, and sharp borders. Vacuolar degeneration was seen in most anterior horn cells at all levels of spinal cord, more so in pup A than in pup B. Vacuolar changes were not seen in other neurons of the spinal cord nor in any neurons in spinal cords of nontransgenic littermates. A loosening of neuropil in the vicinity of the vacuolated perikarya may have obscured the presence of vacuolar changes in the neurites of motor neurons. A loosening of neuropil was seen in other regions of spinal cord and in spinal cords of nontransgenic newborn mice. The remarkable preservation of nuclear detail, however, attested to the structural preservation of neuronal cell bodies in immersion-fixed, paraffin-embedded tissues. The large, round nuclei of vacuolated motor neurons had sharply defined nuclear borders, displayed a finely granular chromatin pattern, and often contained very prominent nucleoli. The same nuclear details were

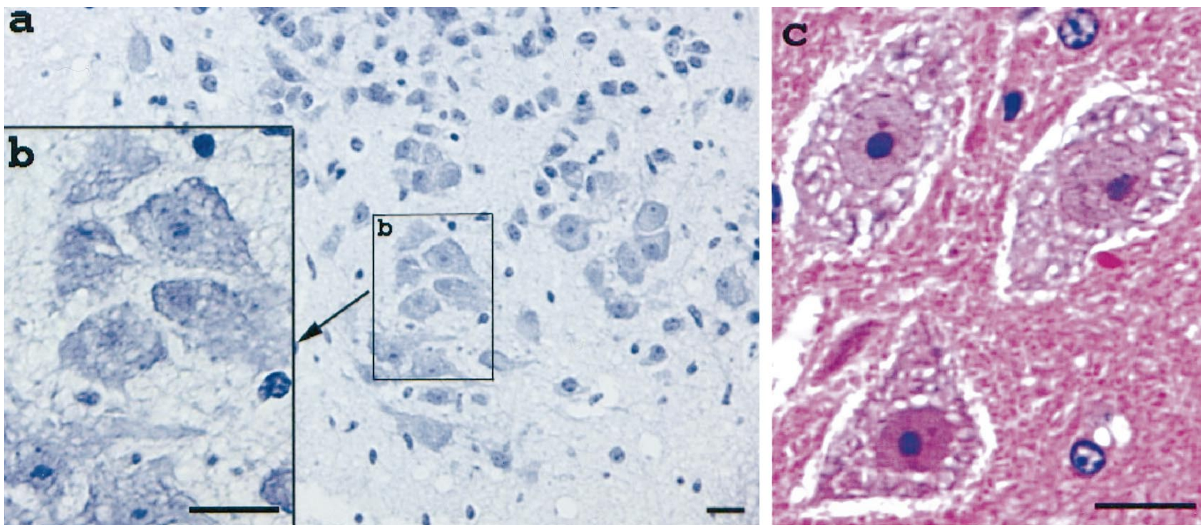

Figure 2. *a*, A cluster of anterior horn cells in the lumbar spinal cord of pup A is enlarged (*b*) to reveal the cytoplasmic vacuolar degeneration of motor neurons. The nuclei of vacuolated neurons contain prominent nucleoli. *c*, Similar vacuolated changes are present in a cluster of three motor neurons of founder mouse D. Stained with hemotoxylin alone (*a*, *b*) or with eosin (*c*). Scale bars: *a*, 75 μm; *b*, *c*, 25 μm.

observed in vacuolated motor neurons of older transgenic mice without a loosening of the surrounding neuropil (Fig. 2*c*).

Immunoreactivities to mouse NF-L and to the human *c-myc* tag in vacuolated and control motor neurons of newborn mice were tested with increasing concentrations of primary antibodies to mouse NF-L and the *c-myc* tag. End products were observed only at titers that produced extensive nonspecific staining of the tissues. When the spinal cords of newborn mice were examined with highly sensitive antibodies to phosphorylated epitopes on the NF-H and NF-M subunits, immunoreactivity was detected in white matter tracts along the dorsal and ventral surfaces of spinal cord, as previously described (Carden et al., 1987). Focal NF accumulations in cell bodies or nearby neurites of vacuolated motor neurons were not seen in newborn mice or in the spinal cords of older mice. The limited amounts of motor neuron tissue in newborn mice precluded a biochemical assessment of NF-L protein levels by Western blot.

The extensive vacuolar degeneration of motor neurons in newborn mice was associated with perturbations in target organ development. Differences in skeletal muscle development were readily apparent in comparative examinations of muscles at the level of the distal tibia from transgenic and nontransgenic newborn pups. Whereas the muscle of newborn controls was composed of uniform bundles of muscle fibers with occasional central nuclei (Fig. 3*b*), the muscle from the transgenic pups A and B contained numerous small cells without myofibrils interspersed among large fibers with myofibrils (Fig. 3*a*). The large fibers had large and hyperchromatic central nuclei that often were associated with perinuclear vacuoles. The features resemble those described during muscle development lacking neurogenic input and have been attributed to a persistence and degeneration of primary myotubes and deficiency of secondary myotube development (Ontell et al., 1988; Condon et al., 1990). Differences between transgenic and nontransgenic muscle were less apparent in musculature from the proximal limbs and along the axial skeleton, suggesting that the changes may reflect a preferential involvement of distal musculature or, possibly, a delay in muscle development.

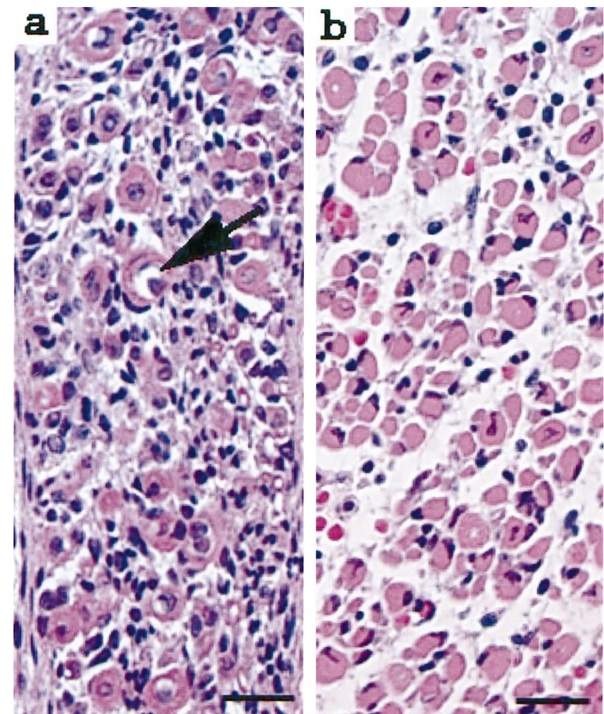

Figure 3. *a*, Cross section of skeletal muscle at the level of the distal tibia from transgenic pup A showing large myofibers with hyperchromatic central nuclei and perinuclear vacuoles (*arrow*) scattered among numerous small cells without myofibrils. *b*, Cross section of skeletal muscle at the level of the distal tibia of a nontransgenic littermate control showing a uniform population of myofibers with central and peripheral nuclei. H&E stain. Scale bars: *a*, *b*, 50 μm.

Transgene expression leads to a stunting of growth during early development

Founder mice and transgenic F1 pups were smaller and less active than their nontransgenic littermates. These traits became apparent during the initial 2–3 week period of postnatal development but did not progress and became less apparent after weaning. Figure 4 shows the stunted growth in an 18 d transgenic F1 pup

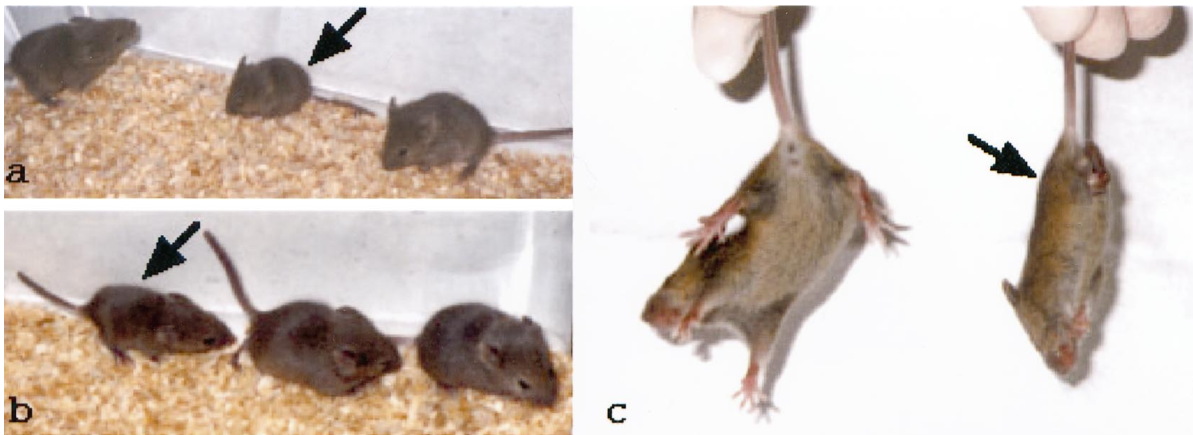

Figure 4. *a, b*, Stunted growth in an 18 d F1 transgenic pup (*arrow*) as compared with two nontransgenic littermates. *c*, The transgenic pup (*arrow*) displays an abnormal reflex of flexing the hind- and forelimbs when held by the tail, as compared with extension of the limbs and writhing movements of a nontransgenic littermate.

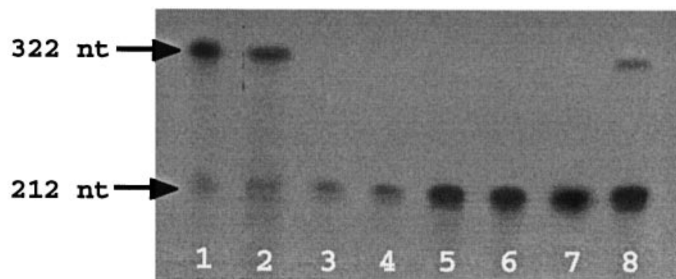

Figure 5. RNA protection assay showing protected fragments of 322 and 212 nt from transgenic and endogenous NF-L mRNAs, respectively, in the brains of pup A (*lane 1*), pub B (*lane 2*), nontransgenic littermates of pups A and B (*lanes 3*, *4*), founder mouse C (*lane 7*), two of her transgenic pups (*lanes 5*, *6*), and founder mouse D (*lane 8*).

(from founder E) as well as the abnormal reflex of flexing the limbs when held by the tail, as previously described (Lee et al., 1994). The mouse did not develop further weakness or paralysis and was killed along with a nontransgenic littermate at 28 d. Microscopic examination revealed a loss of enteric neurons and vacuolar degeneration of motor neurons in the transgenic pup (data not shown). Stunted growth was also a useful phenotypic marker of some, but not all, transgenic pups. When subsequently examined for transgene expression, pups with stunted growth had the highest levels of transgene expression in their respective litters (see below).

Abnormal phenotype correlates with expression of the mutant transgene

The highest levels of transgene expression were found in newborn pups with dilated and malformed intestines. RNA protection assays showed the highest level of transgene expression in the brain of pup A and slightly less in the brain of pup B (Fig. 5), corresponding with the more severe alterations in pup A. Transgene expression was greater than that of endogenous NF-L expression, although the latter is present at very low levels in neonatal rodent brain (Julien et al., 1986; Schlaepfer and Bruce, 1990). Similar levels of NF-L mRNA were noted in newborn transgenic and nontransgenic littermates, indicating that transgene expression did not appear to alter the expression of endogenous NF-L mRNA. Transgene expression was derived from low transgene copy numbers of 2 and 1 in pups A and B, respectively, as estimated by PCR and Southern blot analyses of genomic DNA.

Expression of the transgene in other founder mice and in transgenic lines was quite variable. A female founder (mouse C) transmitted the transgene to four of seven F1 pups, but the transgene was not expressed (Fig. 5) and the abnormal phenotype was not detected in this transgenic line. A male founder (mouse D) was unable to transmit the transgene to three litters of F1 pups. When founder mouse D was killed at 3 months, focal vacuolar degeneration was observed in anterior horn cells (see Fig. 2*c*), and a low level of transgene expression was detected in brain (Fig. 5). Levels of transgene expression also correlated with the severity of neuropathic changes of enteric and motor neurons in the F1 and F2 progeny (see below).

Alterations of enteric and motor neurons occur in transgenic lines

Neuropathic changes of enteric and motor neurons occurred in three transgenic lines (from founders E, F, and G), including eight transgenic pups from the F1 or F2 generations. Two transgenic pups from founder F were less active and without visible milk products in their stomachs, as customarily seen through the thin abdominal wall of newborn suckling mice. When they were killed on day 2, the absence of milk products was confirmed by direct examination. Instead, the stomachs and small intestines, but not the abdominal cavities, were distended with air, as if the pups had attempted to suckle but had ingested air instead of milk. Microscopically, there was extensive depletion of enteric neurons in the small intestine and vacuolar degeneration of motor neurons, similar to that described in founder mice A and B.

In several instances, either one or two newborn F1 pups died during the initial 24 hr postnatal period and probably were cannibalized by the mother so that they were not recovered for genomic typing or examination of the tissues. Nonviability of transgenic pups also was suggested in cross-breeding experiments. Initial cross-breeding of founder mice produced F1 litters with only one or two viable pups and F1 litters with a higher percentage of nontransgenic pups than anticipated. Subsequent cross-breedings of the same founder mice yielded larger F1 litters with close to the anticipated 75% rate of transgene transmission. Transgenic F1 pups, killed at 14 and 28 d, revealed focal losses of enteric neurons and vacuolar changes of motor neurons. The extent of neuropathic changes and corresponding levels of trans-

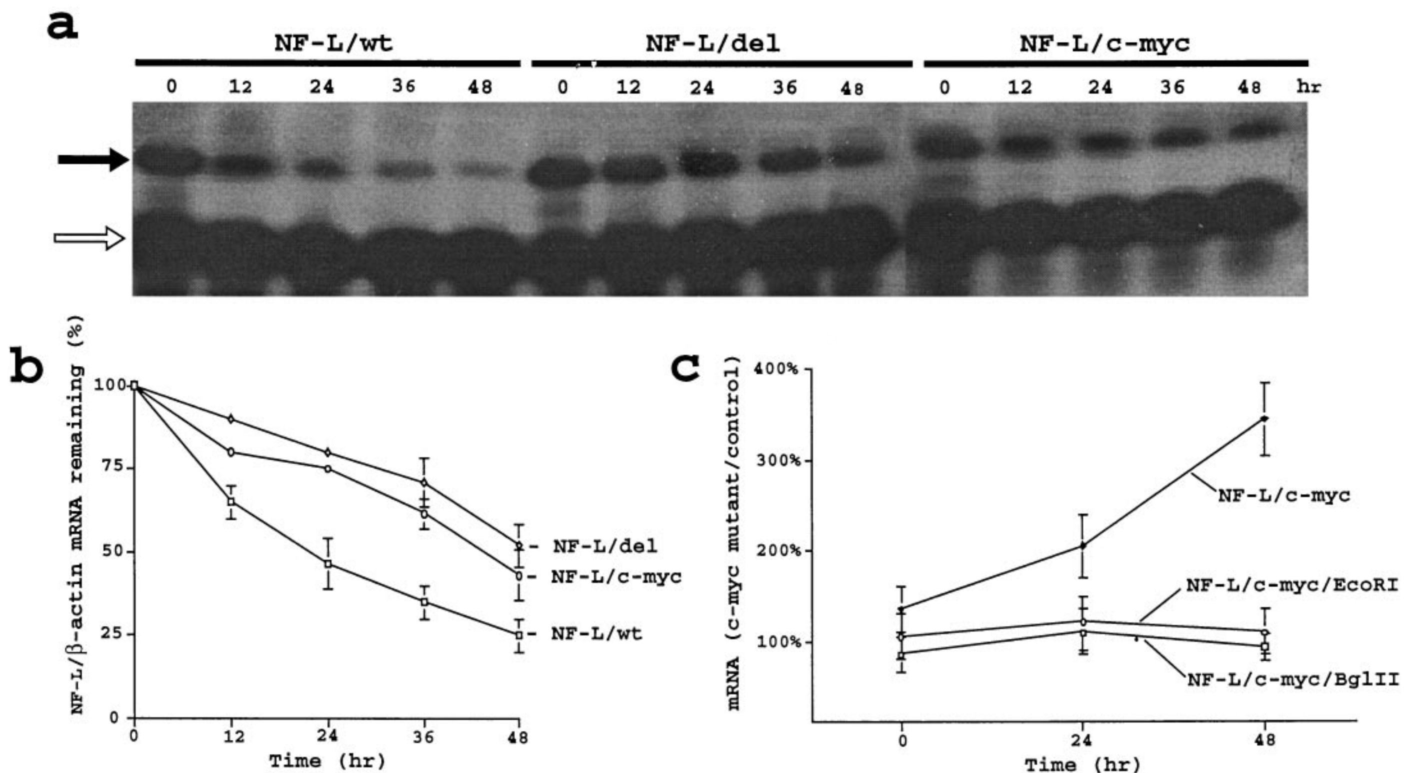

Figure 6. Protection assay (*a*) and quantitation (*b*) of NF-L and β-actin mRNA levels in Neuro 2a cells doubly transfected with tTA transactivator expression vector and target gene in which a Tn-10 tetracycline-inducible promoter drives the expression of wild-type (NF-L/wt) or mutant (NF-L/del and NF-L/*c-myc*) NF-L cDNA. The mutant transgenes either were deleted of 23 bp of distal coding region and 45 bp of proximal 3′-UTR (NF-L/del) or had a 36 bp *c-myc* insert and stop codon appended to the end of the coding region (NF-L/*c-myc*). The NF-L target genes were activated for 72 hr by withdrawal of tetracycline and then inactivated by the readdition of 0.5 μg/ml tetracycline (*0* time point). An RNA protection assay detected radioactive fragments of 125 nt (*filled arrow*) and 83 nt (*open arrow*) from NF-L and β-actin mRNAs. NF-L/β-actin mRNA levels were averaged from three experiments. *c*, mRNA levels from mutant NF-L cDNAs with 36 bp *c-myc* mutation in the *Bgl*II site of exon 1 (NF-L/*c-myc*/*Bgl*II), in the *Eco*RI sites of the distal 3′-UTR (NF-L/*c-myc*/*Eco*RI), and at the end of the coding region (NF-L/*c-myc*). Mutant and wild-type NF-L cDNAs with tetracycline-inducible promoters were cotransfected in Neuro 2a cells, and mRNA levels were assayed by RT-PCR at 0, 24, and 48 hr after inactivation of transcription by the addition of tetracycline. Levels of mRNA from the mutant NF-L cDNAs are expressed as the percentage of mRNA level from the cotransfected wild-type NF-L cDNA.

gene expression were notable less than those observed in founders A and B in the newborn transgenic F1 pups of founder F. Examinations of other tissue, including the kidneys, from the founder mice and transgenic lines were unremarkable.

Our overall findings suggest that the phenotype relates to levels of transgene expression. High-level expression leads to profound neuropathic changes of enteric and motor neurons and is disruptive to perinatal and, possibly, to prenatal viability. Intermediate levels of expression were found in pups with stunted growth. Low-level expression leads to transient dysfunction and limited alterations of enteric and motor neurons.

Insertion of *c-myc* tag disrupts the ability of the stability determinant to regulate the stability of the NF-L transcript

Further studies were undertaken to probe the nature of the *c-myc* mutation on the NF-L transgene. To test the effects of the *c-myc* insert on NF-L mRNA stability, we constructed a full-length NF-L cDNA (NF-L/wt), a cDNA in which 23 bp of distal coding region and 45 bp of proximal 3′-UTR were deleted (NF-L/del), and a cDNA containing a 36 bp *c-myc* insert between the coding region and 3′-UTR (NF-L/*c-myc*). Then the NF-L cDNAs were placed behind a Tn-10 tetracycline-inducible promoter (Gossen and Bujard, 1992) and stably transfected into a neuronal cell line (Neuro 2a) containing the tTA transactivator cDNA under control of an autoinducible promoter (Shockett et al., 1995). The system was shown to be highly inducible when tested with a luciferase reporter gene, generating 1000-fold increases (and decreases) of luciferase activity in the 48 hr interval after withdrawal (and readdition) of tetracycline (Cañete-Soler et al., 1998a).

Stability of mRNAs from the NF-L/wt, NF-L/del, and NF-L/*c-myc* cDNAs was compared by inducing transgene expression for 72 hr in the absence of tetracycline and then measuring mRNA levels at varying time points after the readdition of the ligand. Figure 6*a* shows a representative RNA protection assay of NF-L mRNA (*solid arrow*) and β-actin mRNA (*open arrow*), whereas Figure 6*b* shows the average decline of NF-L/β-actin mRNA levels from four experiments. The NF-L transcript is stabilized either by deleting the entire binding site of the stability determinant (NF-L/del) or by inserting a *c-myc* tag between the 3′-CR and 3′-UTR components of the binding site (NF-L/*c-myc*). The insertion of the *c-myc* tag is almost as effective as the full deletion in disrupting the function of the determinant.

The ability of the *c-myc* insert to alter mRNA stability is attributable to its placement in the stability determinant

To test whether the effect of the 36 bp *c-myc* insert was attributable to the *c-myc* sequence per se or to the context of its place-

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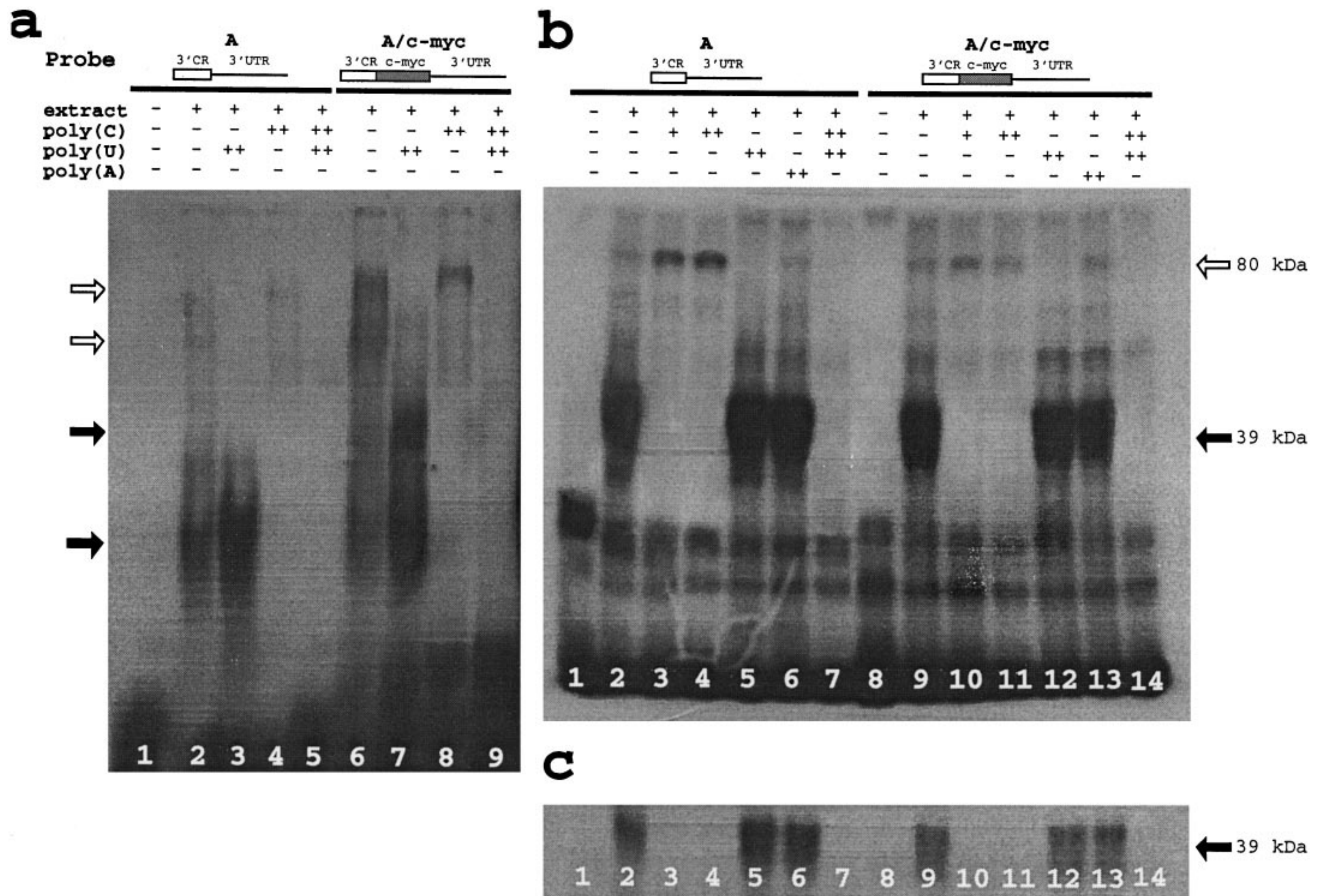

Figure 7. Gel-shift (*a*) and cross-linking (*b*, *c*) assays of RNP complexes that form when brain extracts are incubated with probe A (23 nt of distal coding region and 45 nt of proximal 3′-UTR of NF-L) and probe A/*c-myc* (same probe with *c-myc* tag inserted between the coding region and 3′-UTR). Gel-shift assay (*a*) shows a faster migrating complex (*solid arrows*) competed by poly(C), enhanced by poly(U), and referred to as the C-binding complex. Whereas the C-binding complex primarily is composed of a single band (*lower solid arrow*) on probe A (*lanes 2, 3*), an additional slower migrating band (*upper solid arrow*) forms on probe A/*c-myc* (*lanes 6, 7*). A slower migrating complex (*open arrows*), referred to as the U/A-binding complex, also forms preferentially on the mutant probe. Cross-linking assay (*b, c*) shows that radioactivity from probes A and A/*c-myc* is cross-linked to a major 39 kDa polypeptide (*solid arrow*) and to a minor 80 kDa polypeptide (*open arrow*) and that cross-linkage to the 39 and 80 kDa polypeptides is competed by poly(C) and poly(U), respectively. Cross-linkage of radioactivity from probe A to the 39 kDa polypeptide is enhanced in the presence of poly(U) or poly(A^+) (compare *lanes 5* and *6* with *lane 2* in Fig. 1*c*), but not from probe A/*c-myc* (compare *lanes 12* and *13* with *lane 9* in Fig. 1*c*). Likewise, cross-linkage of radioactivity from probe A to the 80 kDa polypeptide is enhanced in the presence of low (+) or high (++) levels of poly(C) (compare *lanes 3* and *4* with *lane 2*), but not from probe A/*c-myc* (compare *lanes 10* and *11* with *lane 9*). Incubations were conducted with or without extract (160 μg) and at low (20 ng) or high (1 μg) levels of poly(C), poly(U), or poly(A^+) homoribopolymer competitors. A 5% nondenaturing acrylamide gel was used for the gel-shift assay; 10% PAGE was used for cross-linking studies. Molecular weights were estimated by comigration with prestained standards. Figure 1*c* is a briefly exposed autoradiogram of cross-linkage in the 39 kDa polypeptide.

ment in the stability determinant, we assessed the stability of NF-L mRNAs when the *c-myc* insert was placed in exon 1 (NF-L/*c-myc*/*Bgl*II) or in the distal 3′-UTR (NF-L/*c-myc*/*Eco*RI). In both instances, the presence of the *c-myc* insert did not alter the stability of the transcript (Fig. 6*c*). As expected, the stability of the NF-L transcript was enhanced when the *c-myc* tag was inserted into the stability determinant (NF-L/*c-myc*).

The findings indicate that insertion of the *c-myc* tag into the stability determinant of the NF-L mRNA alters the stability of the transcript and that the altered function of the stability determinant is not attributable to the presence of the *c-myc* sequence but to the placement of the *c-myc* tag within the stability determinant. In further support of this interpretation, we find that the 36 bp *c-myc* tag in exon 1 does not gel shift an RNP complex (data not shown) but that placement of the *c-myc* tag at the end of the coding region alters the RNP complexes that form on the major stability determinant of the transcript (see below).

Insertion of the *c-myc* tag alters the RNP complexes that bind to the major stability determinant at the junction of the coding region and 3′-UTR of the NF-L transcript

To test whether insertion of a 36 nucleotide (nt) *c-myc* tag at the junction of coding region and 3′-UTR of NF-L alters the binding of RNP complexes to this site, we undertook gel-shift and cross-linking assays to compare the complexes that form when brain extracts are incubated with probes of wild-type and mutant sequence (Fig. 7). The RNP complexes that assemble on an RNA probe composed of the 23 nt of 3′-CR and the 45 nt of proximal 3′-UTR (Fig. 7*a, lane 2*) consist primarily of a set of bands (*solid*

arrows) that is competed away by poly(C) homoribopolymers (*lanes 4, 5*), enhanced in the presence of poly(U) (*lane 3*), and is referred to as the C-binding complex (Cañete-Soler et al., 1998b). A similar C-binding complex with a slightly different pattern of electrophoretic migration forms on the probe with a *c-myc* tag insert between the 3′-CR and 3′-UTR (*lane 6*). Although the C-binding complex on the mutant probe also is competed with poly(C) (*lanes 8, 9*) and enhanced in the presence of poly(U) (*lane 7*), a large percentage of the complex has a slower rate of electrophoretic migration (*upper solid arrow*), as if forming a larger aggregate. The formation of an additional slower-migrating component in the C-binding complex on the mutant probe was observed consistently in six gel-shift assays by using three different preparations of brain extract that were either freshly prepared or retrieved from storage at −80°C. Formation of slower- and faster-migrating components of the C-binding complex on the mutant probe was observed when gel-shift assays were conducted with 160, 80, 40, 20, or 10 μg of protein. In all instances, at least 35% of radioactivity of the C-binding complex was present in the slower-migrating band.

Insertion of a *c-myc* tag between the coding region and 3′-UTR of the NF-L probe also led to an enrichment of a slow migrating set of bands (*open arrows*) on the mutant probe (*lane 6*) that is competed with poly(U) (*lanes 7, 9*) and is referred to as the U/A-binding complex (Cañete-Soler et al., 1998b). The U/A-binding complex tends to aggregate into a slow-migrating band (*upper open arrow*) when assembled in the presence of poly(C) (*lane 8*). Small amounts of the U/A-binding complex also form on the wild-type probe (*lanes 2, 4*), but always at lower levels than amounts that form on the mutant probe.

When the complexes that form on radioactive probes from brain extracts are cross-linked by UV irradiation, digested, and examined on denaturing SDS gels (Fig. 7*b*), radioactivity from the wild-type (*lane 2*) and mutant (*lane 9*) probes are present in a major 39 kDa polypeptide (*solid arrow*) and a minor 80 kDa polypeptide (*open arrow*). Because the cross-linking to the 39 kDa polypeptides is competed with poly(C) (*lanes 3–4, 10–11*) and the cross-linking to the 80 kDa polypeptide is competed with poly(U) (*lanes 5, 12*), they are interpreted as core-binding components of the C-binding and U/A-binding complexes, respectively (Cañete-Soler et al., 1998b). Although radioactivities from the wild-type and mutant probes are cross-linked to the same core-binding polypeptides, there are small differences in the amounts of cross-linked radioactivity, especially when formations of the C- or U/A-binding complexes are competed with poly(C) or poly(U). For example, cross-linking to the 80 kDa polypeptide is enhanced in the presence of poly(C) when the wild-type probe is used (compare *lane 2* with *lanes 3* and *4*), but not when the mutant probe is used (compare *lane 9* with *lanes 10* and *11*). Likewise, the addition of poly(U) or poly(A^+) enhances the cross-linking to the 39 kDa polypeptide from the wild-type probe (compare *lane 2* with *lanes 5* and *6*, Fig. 7*c*), but not from the mutant probe (compare *lane 9* with *lanes 12* and *13*, Fig. 7*c*). The enhanced cross-linking to core binding polypeptides when formations of C- and U/A-binding complexes are competed is a characteristic feature of wild-type probes (Cañete-Soler et al., 1998b). The lack of a corresponding enhancement in the cross-linking to core binding components from the mutant probe was observed consistently.

Cross-linkage of radioactivity to the 39 kDa polypeptide from the mutant probe was also less than that from the wild-type probe (compare *lane 2* with *lane 9*, Fig. 7*c*). To determine whether the reduction in cross-linkage was attributable to differences in probe concentration, we conducted cross-linkage studies with increasing amounts of probes that were diluted to the same specific radioactivities. The results indicate that the *c-myc* mutation leads to a twofold reduction in cross-linkage to the 39 kDa polypeptide over a wide range of probe concentrations (data not shown).

In summary, gel-shift and cross-linking studies show that very similar C-binding and U/A-binding complexes form on the wild-type and mutant sequences but that the relative amounts, electrophoretic migrational rates, and putative interactions of C-binding and U/A-binding RNP components are altered by insertion of the *c-myc* tag. The biochemical alterations produced by the *c-myc* mutation are not dramatic but are reproducible. They could reflect unknown facets of the RNP interactions that regulate NF-L mRNA stability and mediate the neuropathic effects of the mutant transgene.

DISCUSSION

The *c-myc* mutation in the NF-L transgene produces a novel phenotype

The results of this study show that a 36 bp *c-myc* insert at the end of the coding region in a NF-L transgene is sufficient to cause profound alterations of the enteric nervous system as well as vacuolar degeneration of motor neurons in transgenic mice. The phenotype differs from that previously reported from a NF-L transgene with the same *c-myc* mutation and a leucine-to-proline mutation in the rod domain of the protein (Lee et al., 1994). The differences in phenotype are not trivial and could be attributable to additional or separate effects from the point mutation in the transgene. It is also possible that varying phenotypes could reflect the use of different promoters. For example, use of a murine sarcoma virus promoter (Lee et al., 1994) may have favored transgene expression in motor neurons at levels or developmental periods in which the neurons are particularly susceptible to the adverse effects of the transgene. The use of a human CMV promoter in the present study may have generated a different pattern of transgene expression, enhancing transgene expression in enteric neurons and altering levels or time course of transgene expression in motor neurons. Levels of transgene expression that were not disruptive to the viability of transgenic pups may have been insufficient to cause more than a transient dysfunction of motor neurons. Alternatively, there may have been diminishing levels of transgene expression in motor neurons during postnatal development.

Mice bearing the NF-L transgene with only the *c-myc* mutation also displayed a stunting of growth, as previously reported in mice bearing the mutant (Lee et al., 1994) and wild-type (Xu et al., 1993) NF-L transgene. The occurrence of stunted growth with transient motor neuron dysfunction indicates that the phenomenon does not necessarily occur *pari passu* with progressive motor weakness and paralysis but reflects a separate effect of mutant transgene expression. The transient appearance of stunted growth during a period of rapid maturation of intestinal function (Traber and Silberg, 1996) raises the possibility that the phenomenon may relate to alterations of the enteric nervous system and a temporary disruption of intestinal function.

The neuropathic effects of a NF transgene on the enteric nervous system have not been reported previously. The findings suggest an inherent susceptibility of developing enteric neurons to expression of the mutant transgene or a disproportional level of transgene expression in the enteric nervous system. Because the *c-myc* mutation stabilizes the NF-L mRNA, variability in the

stabilizing process could lead to disparate levels of transgene expression in different neuronal tissues at varying stages of development. Replacement of the 3′-UTR of a *Hox-1.4* transgene leads to a marked and selective increase of transgene expression in the embryonic gut and to a profound developmental malformation of the distal colon in transgenic mice (Wolgemuth et al., 1989). It is also possible that the use of a different promoter favors the expression of the mutant NF-L transgene in enteric neurons.

Vacuolar degeneration of motor neurons is characteristic of other transgenic models of motor neuron degeneration (DalCanto and Gurney, 1995; Wong et al., 1995b) but has not been described previously in mice bearing a NF transgene. It is unclear, however, whether the nature of pathological changes is a reliable measure for distinguishing transgenic models of motor neuron degeneration. For example, vacuolar changes (DalCanto and Gurney, 1995; Wong et al., 1995b) as well as NF accumulations (Tu et al., 1996) can occur in the same transgenic model of motor neuron degeneration and may or may not relate to the immediate cause of neuronal degeneration. Moreover, the massive vacuolar degeneration, because of swollen mitochondria, coincides with the onset of neuronal dysfunction and precedes the loss of motor neurons or their axons (Kong and Xu, 1998). A similar pattern of motor neuron pathology could explain the extensive vacuolar degeneration without apparent loss of motor neurons in newborn mice expressing high levels of a mutant NF-L transgene. In the latter instance an assessment of the natural sequelae of extensive vacuolar degeneration is precluded by the lethal effects of the transgene. The presence of focal vacuolar changes in motor neurons of asymptomatic founder mice suggests that the changes may even be transient and, possibly, reversible. Changes of mitochondrial membrane permeability represent a very early stage of apoptosis as well as cell necrosis (see Kroemer et al., 1998) so that vacuolar changes could reflect a very early pathological alteration to which motor neurons are highly susceptible.

Alterations in RNA processing are implicated in the pathogenesis of neuronal degeneration

The neuropathic effects of NF transgene have been attributed to alterations at the level of protein expression rather than to alterations at other levels of transgene expression. This perspective is exemplified by the view that neuronal degeneration is secondary to the disruption of NF assembly, axonal transport, or other cytoskeletal function (Collard et al., 1995; Bruijn and Cleveland, 1996). From this perspective the neuropathic effects of the *c-myc* insert might be attributed to the stabilization of NF-L mRNA and the consequent increased expression of NF-L protein, because increased levels of NF-L protein are known to have neuropathic effects (Xu et al., 1993). This explanation would require an increased expression of NF-L protein and could not account for the enhanced neuropathic effect of a NF-L transgene with the *c-myc* mutation that occurs at reduced levels of NF-L protein (Lee et al., 1994). We were also unable to detect any increase of transgene expression at the protein level in vacuolated motor neurons. Moreover, our findings show that the neuropathic effects of the *c-myc* mutation occur during a perinatal (or prenatal) period in which there is very limited expression of endogenous NF subunits (Julien et al., 1986; Schlaepfer and Bruce, 1990) and before the establishment of any known NF function. It therefore seems unlikely that aberrant expression of NF-L protein during early neuronal development would have neuropathic effects because of the disruption of NF or cytoskeletal function. Very high levels of NF-L protein can accumulate in non-neuronal (e.g., skeletal muscle and kidney) tissues of transgenic mice without apparent pathological effects (Monteiro et al., 1990). Moreover, complete loss of NF-L expression because of ablation of the gene in mice (Zhu et al., 1997) or because of a spontaneous mutation in quail (Yamasaki et al., 1992) does not affect the viability of motor neurons or any other subset of neurons.

An alternative mechanism for the neuropathic effects of the *c-myc* mutation is that they are attributable to expression of the mutant NF-L mRNA, but not because of the stabilization of mRNA and increased expression of NF-L protein. Instead, the disruptive effects could be attributable to the presence of mutant NF-L mRNA as a substrate for the binding of RNP complexes that mediate post-transcriptional processing of NF-L and other gene products. According to this view, the addition of exogenous NF-L mRNA serves to titrate or alter the RNP complexes with which they interact. If the same RNP complexes mediate post-transcriptional processing of NF-L and other neuronal mRNAs, a titration of the RNP complexes could affect the expression of the other neuronal gene products adversely. Moreover, if the *c-myc* mutation alters the binding of RNP complexes to the NF-L mRNA, this alteration could be important in mediating the enhanced neuropathic effects or "gain-of-adverse function" that is conferred by the *c-myc* mutation to the NF-L transgene.

Our working hypothesis is that the NF-L transgene, by titrating RNP complexes, disrupts the expression of gene products that maintain neuronal homeostasis, such as those that override apoptosis (see Easton et al., 1997). Pathways that override apoptosis are acquired during the same postnatal interval in which there is a marked upregulation of NF mRNAs (Schwartz et al., 1990) because of a stabilization of the NF transcripts (Schwartz et al., 1994). The axotomy-induced destabilization of NF mRNAs (Schwartz et al., 1992) indicates that the pathways regulating the stability of NF transcripts are responsive to disruption of neuronal homeostasis and remain operational in mature neurons. Furthermore, both the postnatal stabilization and the axotomy-induced destabilization of the NF-L mRNA are regulated by determinants in the 3′-UTR of the NF-L gene (Schwartz et al., 1995). Studies are currently underway to identify the components of RNP complexes that regulate the stability of the NF-L transcript and to uncover other gene products with which they interact.

The ability of the *c-myc* mutation to confer a gain-of-adverse function to the mutant NF-L transgene directs attention to the stability determinant as the likely binding site of RNP complex implicated in the neuropathic effects of the transgene. Further implications of the C-binding complex in the neuropathic effects derive from the fact that this complex assembles on transcripts of each NF transgene that has neuropathic effects in transgenic mice. Indeed, the same C-binding complex binds to the stability determinant of the mouse NF-L mRNA and to the 3′-UTR of the human NF-H mRNA, but not to the 3′-UTR of mouse NF-M (R. Cañete-Soler, unpublished data). These results could explain the neuropathic effects that occur from overexpression of the mouse NF-L (Xu et al., 1993) and human NF-H (Cote et al., 1993) transgenes and the apparent lack of neuropathic effects from overexpression of the mouse NF-M (Wong et al., 1995a) or mouse NF-H (Marszalek et al., 1996) transgenes. The gain-of-adverse effects imparted by the *c-myc* mutation suggest that the neuropathic effects may relate to the titration or alteration of component(s) within the C-binding complex rather than to titration of the entire complex.

Post-transcriptional regulation is important in determining and maintaining neuronal phenotype

The complexities of neuronal development take place entirely in postmitotic neurons. It therefore follows that developing neurons are deprived of the global reorganization of chromatin that occurs at cell division and may be limited in their opportunity to regulate gene activity at the level of transcription. Under these circumstances there may have evolved a greater reliance on post-transcriptional mechanisms for establishing neuronal identity and maintaining their viability among the vast arrays of neuronal subsets. Strongly conserved families of neuron-specific RNA-binding proteins have been identified that (1) are expressed sequentially during neuronal differentiation (Wakamatsu and Weston, 1997), (2) are expressed specifically in subsets of neurons (Okano and Darnell, 1997), (3) are required for the development and maintenance of neurons (Yao et al., 1993), and (4) are implicated in the selective degeneration of different neuronal subsets in paraneoplastic neurological syndromes (Szabo et al., 1991; Manley et al., 1995; Darnell, 1996). Although members of the Hu and Elav families bind to AU-rich sequence of neuronal transcripts (Gao et al., 1994) and participate in the regulation of splicing (Koushika et al., 1996), mRNA stability (Tsai et al., 1997; Fan and Steitz, 1998; Levy et al., 1998; Peng et al., 1998), and translation (Antic and Keene, 1998), there is limited information as to their role in maintaining neuronal viability (see Yao et al., 1993).

There is, however, evidence that RNA processing is involved in the pathogenesis of neurodegenerative diseases, including motor neuron disease. The mutations responsible for spinal muscular atrophy (Lefebvre et al., 1995, 1997) as well as Fragile X syndrome (Verkerk et al., 1991) occur in genes of RNA-binding proteins (Siomi et al., 1993; Liu and Dreyfuss, 1996). The SMN gene product is part of a large complex involved in spliceosomal snRNP biogenesis and function (Liu et al., 1997). Aberrant splicing of RNA recently has been implicated in sporadic amyotrophic lateral sclerosis, albeit in glial supporting cells (Lin et al., 1998). In myotonic dystrophy, expression of a mutant mRNA leads to abnormal splicing of a wild-type gene product, termed a trans-dominant effect (Philips et al., 1998). In the latter instance, a CUG expansion in the 3′-UTR of the mutant protein kinase mRNA increases the binding of a CUG-binding protein (CUG-BP) and promotes alternative splicing of troponin T RNA. A similar trans-dominant effect of a mutant RNP-binding site could be responsible for the adverse effects of the *c-myc* mutation in the NF-L mRNA, as described in this report.

The present study is the first direct causal linkage between an alteration in RNA processing and a neurodegenerative state in transgenic mice. A transgenic model thereby is established for probing the aberrant pathways leading to a neurodegenerative phenotype. At the same time, the findings underscore the importance of post-transcriptional regulation in the pathogenesis of a neurodegenerative state. Further insights into the post-transcriptional mechanisms underlying neurodegeneration in transgenic mice undoubtedly will advance our understanding of neurodegenerative diseases.

REFERENCES

Antic D, Keene JD (1998) Messenger ribonucleoprotein complexes containing human ELAV proteins: interactions with cytoskeleton and translational apparatus. J Cell Sci 111:183–197.

Bruijn LI, Cleveland DW (1996) Mechanisms of selective motor neuron death in ALS: insights from transgenic mouse models of motor neuron disease. Neuropathol Appl Neurobiol 22:373–387.

Cañete-Soler R, Schwartz ML, Hua Y, Schlaepfer WW (1998a) Stability determinants are localized to the 3′UTR and 3′ coding region of the neurofilament light subunit mRNA using a tetracycline-inducible promoter. J Biol Chem 273:12650–12654.

Cañete-Soler R, Schwartz ML, Hua Y, Schlaepfer WW (1998b) Characterization of ribonucleoprotein complexes and their binding sites on the neurofilament light subunit (NF-L) mRNA. J Biol Chem 273:12655–12661.

Carden MJ, Trojanowski JQ, Schlaepfer WW, Lee VML (1987) Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. J Neurosci 7:3489–3504.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Collard J-F, Cote F, Julien JP (1995) Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. Nature 375:61–64.

Condon K, Silberstein L, Blau HM, Thompson WJ (1990) Differentiation of fiber types in aneural musculature of the prenatal rat hindlimb. Dev Biol 138:275–295.

Cote F, Collard JF, Julien JP (1993) Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. Cell 73:35–46.

Couillard-Despres S, Zhu Q, Wong P, Price DL, Cleveland DW, Julien JP (1998) Protective effect of neurofilament heavy gene overexpression in motor neuron disease induced by mutant superoxide dismutase. Proc Natl Acad Sci USA 95:9626–9630.

DalCanto MC, Gurney ME (1995) Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing wild-type human SOD: a model of familial amyotrophic lateral sclerosis (FALS). Brain Res 676:25–40.

Darnell RB (1996) Onconeural antigens and the paraneoplastic neurological disorders: at the intersection of cancer, immunity, and the brain. Proc Natl Acad Sci USA 93:4529–4536.

Easton RM, Deckworth TL, Parsadanian AS, Johnson EM (1997) Analysis of the mechanism of loss of trophic factor dependence associated with neuronal maturation: a phenotype indistinguishable from *Bax* deletion. J Neurosci 17:9656–9666.

Eyer J, Cleveland DW, Wong PC, Peterson AC (1998) Pathogenesis of two axonopathies does not require axonal neurofilaments. Nature 391:584–587.

Fan XC, Steitz JA (1998) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the *in vivo* stability of ARE-containing mRNAs. EMBO J 17:3448–3460.

Gao FB, Carson CC, Levine T, Keene JD (1994) Selection of a subset of mRNAs from combinatorial 3′-untranslated region libraries using neuronal RNA-binding protein Hel-N1. Proc Natl Acad Sci USA 91:11207–11211.

Gill SR, Wong PC, Monteiro MJ, Cleveland DW (1990) Assembly properties of dominant and recessive mutations in the small mouse neurofilament (NF-L) subunit. J Cell Biol 111:2005–2019.

Gossen MJ, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551.

Julien JP, Meyer D, Flavell D, Hurst J, Grosvelt FG (1986) Cloning and developmental expression of the murine neurofilament gene family. Mol Brain Res 1:243–250.

Karaosmanoglu T, Aygun B, Wade PR, Gershon MD (1996) Regional differences in the numbers of neurons in the myenteric plexus of the guinea pig small intestine and colon: an evaluation of markers used to count neurons. Anat Rec 244:470–480.

Kong J, Xu Z (1998) Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD-1. J Neurosci 18:3241–3250.

Koushika SP, Lisbin MH, White K (1996) ELAV, a *Drosophila* neuron-specific protein, mediates the generations of an alternatively spliced neuronal protein isoform. Curr Biol 6:1634–1641.

Kroemer G, Dallaporta B, Resche-Rigon M (1998) The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol 60:619–642.

Lee MK, Xu Z, Wong PC, Cleveland DW (1993) Neurofilaments are obligate heteropolymers *in vivo*. J Cell Biol 122:1337–1350.

Lee MK, Marszalek JR, Cleveland DW (1994) A mutant neurofilament

subunit causes massive, selective motor neuron death: implications for the pathogenesis of human motor neuron disease. Neuron 13:975–988.

Lee VML, Carden MJ, Schlaepfer WW, Trojanowski JQ (1987) Monoclonal antibodies distinguish several differentially phosphorylated states of the two largest rat neurofilament subunits (NF-H and NF-M) and demonstrate their existence in the normal nervous system of adult rats. J Neurosci 7:3474–3488.

Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M, LePaslier D, Frezal J, Cohen D, Weissenback J, Munnich A, Milki J (1995) Identification and characterization of a spinal muscular atrophy-determining gene. Cell 80:155–165.

Lefebvre S, Burlet P, Liu Q, Bertrandy S, Clermont O, Munnich A, Dreyfuss G, Melki J (1997) Correlation between severity and SMN protein level in spinal muscular atrophy. Nat Genet 16:265–269.

Levy N, Chung SM, Furneaux H, Levy AP (1998) Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J Biol Chem 273:6417–6423.

Lin CLG, Bristol LA, Jin L, Dykes-Hoberg M, Crawford Clawson L, Rothstein JD (1998) Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. Neuron 20:589–602.

Liu Q, Dreyfuss G (1996) A novel nuclear structure containing the survival of motor neurons protein. EMBO J 15:3555–3565.

Liu Q, Fischer U, Wang F, Dreyfuss G (1997) The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. Cell 90:1013–1021.

Manley GT, Smitt PS, Dalmau J, Posner JB (1995) Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites. Ann Neurol 38:102–110.

Marszalek JR, Williamson TL, Lee MK, Xu Z, Hoffman PN, Becher MW, Crawford TO, Cleveland DW (1996) Neurofilament subunit NF-H modulates axonal diameter by selectively slowing neurofilament transport. J Cell Biol 135:711–724.

Monteiro MJ, Hoffman PN, Gearhart JD, Cleveland DW (1990) Expression of NF-L in both neuronal and nonneuronal cells of transgenic mice: increased neurofilament density in axons without affecting caliber. J Cell Biol 111:1543–1557.

Okano JH, Darnell RB (1997) A hierarchy of Hu RNA binding proteins in developing and adult neurons. J Neurosci 17:3024–3037.

Ontell M, Hughes D, Bourke D (1988) Morphometric analysis of the developing mouse soleus muscle. Am J Anat 181:279–288.

Peng SSY, Chen CY, Xu N, Shyu AB (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J 17:3461–3470.

Philips AV, Timchenko LT, Cooper TA (1998) Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. Science 280:737–741.

Schlaepfer WW, Bruce J (1990) Simultaneous up-regulation of neurofilament proteins during the postnatal development of the rat nervous system. J Neurosci Res 25:39–49.

Schwartz ML, Shneidman PS, Bruce J, Schlaepfer WW (1990) Axonal dependency of the postnatal upregulation in neurofilament expression. J Neurosci Res 27:193–201.

Schwartz ML, Shneidman PS, Bruce J, Schlaepfer WW (1992) Actinomycin prevents the destabilization of neurofilament mRNA in primary sensory neurons. J Biol Chem 267:24596–24600.

Schwartz ML, Shneidman PS, Bruce J, Schlaepfer WW (1994) Stabilization of neurofilament transcripts during postnatal development. Mol Brain Res 27:215–220.

Schwartz ML, Bruce J, Shneidman PS, Schlaepfer WW (1995) Deletion of 3′-untranslated region alters the level of mRNA expression of a neurofilament light subunit transgene. J Biol Chem 270:26364–26369.

Shockett P, Difilippantonio M, Hellman N, Schatz DG (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. Proc Natl Acad Sci USA 92:6522–6526.

Siomi H, Siomi MC, Nussbaum RL, Dreyfuss G (1993) The protein product of fragile X gene, FMR1, has characteristics of an RNA-binding protein. Cell 74:291–298.

Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, Henson H, Posner JB, Furneaux HM (1991) HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to *Elav* and *Sex-lethal*. Cell 67:325–333.

Traber PG, Silberg DG (1996) Intestine-specific gene transcription. Annu Rev Physiol 58:275–297.

Tsai KC, Cansino VV, Kohn DT, Neve RL, Perrone-Bizzozero NI (1997) Post-transcriptional regulation of the GAP-43 gene by specific sequences in the 3′-untranslated region of the mRNA. J Neurosci 17:1950–1968.

Tu PH, Raju P, Robinson KA, Gurney ME, Trojanowski JQ, Lee VML (1996) Transgenic mice carrying a human superoxide dismutase transgene develop neuronal cytoskeletal pathology resembling human amyotrophic lateral sclerosis lesions. Proc Natl Acad Sci USA 93:3155–3160.

Verkerk AJMH, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DPA, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang F, Eussen BE, VanOmmen GJB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT, Nelson DL, Oostra BA, Warren ST (1991) Identification of a gene (FMR1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905–914.

Wakamatsu Y, Weston JA (1997) Sequential expression and role of Hu RNA-binding proteins during neurogenesis. Development 124:3449–3460.

Williamson TL, Bruijn LI, Zhu Q, Anderson KL, Anderson SC, Julien JP, Cleveland DW (1998) Absence of neurofilaments reduces the selective vulnerability of motor neurons and slows disease caused by a familial amyotrophic lateral sclerosis-linked superoxide dismutase-1 mutant. Proc Natl Acad Sci USA 95:9631–9636.

Wolgemuth DJ, Behringer RR, Mostsoller NP, Brinster RL, Palmiter RD (1989) Transgenic mice overexpressing the mouse homeobox-containing gene *Hox-1.4* exhibit abnormal gut development. Nature 337:464–467.

Wong PC, Cleveland DW (1990) Characterization of dominant and recessive assembly-defective mutations in mouse neurofilament NF-M. J Cell Biol 111:1987–2003.

Wong PC, Marszalek J, Crawford TO, Xu Z, Hsieh ST, Griffin JW, Cleveland DW (1995a) Increasing neurofilament subunit NF-M expression reduces axonal NF-H, inhibits radial growth, and results in neurofilamentous accumulation in motor neurons. J Cell Biol 130:1413–1422.

Wong PC, Pardo CA, Borchelt DR, Lee MK, Copeland NG, Jenkins NA, Sisodia SS, Cleveland DW, Price DL (1995b) An adverse property of a familial ALS-linked SOD-1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. Neuron 14:1105–1116.

Xu Z, Cork LC, Griffin JW, Cleveland DW (1993) Increased expression of neurofilament subunit NF-L produces morphological alterations that resemble the pathology of human motor neuron disease. Cell 73:23–33.

Yamasaki H, Bennett GS, Itakura C, Mizutani M (1992) Defective expression of neurofilament protein subunits in hereditary hypotrophic axonopathy of quail. Lab Invest 66:734–743.

Yao KM, Samson ML, Reeves R, White K (1993) Gene *elav* of *Drosophila melanogaster*: a prototype for neuronal-specific RNA binding protein gene family that is conserved in flies and humans. J Neurobiol 24:723–739.

Zhu Q, Couillard-Despres S, Julien JP (1997) Delayed maturation of regenerating myelinated axons in mice lacking neurofilaments. Exp Neurol 148:299–316.