Characterization of Ribonucleoprotein Complexes and Their Binding Sites on the Neurofilament Light Subunit mRNA*

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Levels of neurofilament (NF) gene expression are important determinants of basic neuronal properties, but overexpression can lead to motoneuron degeneration in transgenic mice. In a companion study (Cañete-Soler, R., Schwartz, M. L., Hua, Y., and Schlaepfer, W. W. (1998) J. Biol. Chem. 273, 12650–12654), we show that levels of NF expression are regulated by altering mRNAs and that stability determinants are present in the 3’-coding region (3’-CR) and 3’-untranslated region (3’-UTR) of the NF light subunit (NF-L) transcript. This study characterizes the ribonucleoprotein complexes that bind to the NF-L mRNA when cytoplasmic brain extracts are incubated with radioactive probes. Gel retardation assays reveal ribonucleoprotein complexes that are selectively competed with poly(C) or poly(U)/poly(A) homoribopolymers and are referred to as C-binding and U/A-binding complexes, respectively. The C-binding complex forms on the proximal 45 nucleotides of 3’-UTR, but its assembly is markedly enhanced by 22 nucleotides of flanking 3’-CR sequence. U/A-binding complexes form at multiple binding sites in the 3’-CR and 3’-UTR. A pattern of reciprocal binding suggests that the C-binding and U/A-binding complexes interact and may compete for common components or binding sites. Cross-linking studies reveal unique polypeptides in the C-binding and U/A-binding complexes. The findings provide the basis for probing mechanisms regulating NF-L mRNA stability and the relationship between NF overexpression and motoneuron degeneration in transgenic mice.

NF up-regulation, are determined by the nature of target cell innervations and can be reconstituted in transected nerve upon successful reinnervation of the target site (6). Moreover, the stability of NF mRNAs becomes dependent upon continuity of axons with target sites in that the transcripts are destabilized upon nerve transection or excision and transfer of parent neurons to primary culture (7).

Interestingly, NF mRNAs are stabilized during the same postnatal interval in which neurons acquire the ability to survive nerve transection and mount a regenerative response (8). The overlapping appearance of these phenomena raises the possibility that common components of posttranscriptional pathways regulate neuronal homeostasis and levels of NF expression. If so, then titration of regulatory components could account for: (i) the motoneuron degeneration in transgenic mice upon overexpression of a wild-type NF-L (9) or NF-H (10) transgene or modest expression of a mutant NF-L transgene (11), (ii) the preferential degeneration of NF-enriched neurons in mice bearing a mutant SOD-1 transgene (12), and (iii) the transient up-regulation of NF mRNA that precedes the spontaneous motoneuron in Wobbler mice (13). Mechanisms regulating levels of NF expression may therefore bear an important relationship to neurodegenerative states.

We have begun to map stability determinants that regulate steady-state levels of NF-L mRNA. Deletion of the 3’-untranslated region (3’-UTR) from a mouse NF-L transgene stabilizes the NF-L transcript in neuronal cell lines and alters the developmental up-regulation and axotomy-induced down-regulation of the transgene in transgenic mice (14). More recently, we have begun to apply an expression system with tetracycline-regulated promoter to map stability determinants in NF mRNAs. In a companion study (1), we demonstrate the presence of stability determinants in the 3’-coding region (3’-CR) and 3’-UTR of NF-L mRNA and have localized a stabilizing element at the junction of 3’-CR and 3’-UTR that may account for the stabilizing properties of the transcript.

The present study examines the ribonucleoprotein (RNP) complexes that assemble on NF-L mRNA when nascent radiolabeled fragments of the transcript are incubated with cytosolic brain extracts. The RNP complexes have been characterized by their abilities to be competed with homoribopolymers, thereby taking advantage of the high affinity and selective binding of many RNA-binding proteins for specific homoribopolymers (15). A similar approach has been very helpful in characterizing RNP complexes that are believed to stabilize α-globin mRNA (16, 17). The goal of the present study is to correlate biochemical with functional studies (1) and thereby develop a working model for elucidating the mechanisms that regulate the stability of the mouse NF-L transcript.

**EXPERIMENTAL PROCEDURES**

Preparation of Cytosolic Extracts—Rat brain and liver (10–15 g) were minced, washed in phosphate-buffered saline, and homogenized in...
Fig. 1. Schematic diagram of RNA probes in relationship to the mouse NF-L cDNA. Nucleotides are numbered according to the transcription start site.

Binding of RNP Complexes to the NF-L mRNA

poly(G). The faster migrating bands are referred to as the C-binding complex, whereas the slower migrating bands are poly(U), but not by 100-fold higher concentrations of poly(G), but not by 10-fold higher concentrations of poly(C). The faster migrating bands are referred to as the C-binding complex, whereas the slower migrating bands are poly(U), but not by 10-fold higher concentrations of poly(C) or poly(U). Similarly, a set of slower migrating bands (lanes 13–15) were competed by 200 ng of poly(A) or poly(U). The faster migrating bands were competed by 200 ng of poly(A) or poly(U). The faster migrating bands were not seen when the probes were incubated without extract (lane 7)

RESULTS

RNP Complexes Bind to the NF-L mRNA and Are Selectively Competed with Specific Homoribopolymers—An NF-L mRNA probe (A680) that spans the entire 3'-UTR (427 nt) and distal 253 nt of 3'-CR (see Fig. 1) was used to identify binding factors and binding sites that could account for stability determinants in these regions of the transcript (1). When brain extracts were incubated with the radioactive A680 probe and electrophoresed on non-denaturing gels, multiple gel-shifted bands were detected (Fig. 2, lane 3). These bands were characterized by their abilities to be competed by addition of increasing amounts of poly(C) (lanes 4–6), poly(U) (lanes 7–9), poly(A) (lanes 10–12), or poly(G) (lanes 13 and 14) homoribopolymers. A set of faster migrating bands (solid arrowheads) were competed by 20 ng of poly(C), but not by 100-fold higher concentrations of poly(G), poly(A), or poly(U). Similarly, a set of slower migrating bands (open arrowheads) were competed by 200 ng of poly(A) or poly(U), but not by 10-fold higher concentrations of poly(C) or poly(G). The faster migrating bands are referred to as the C-binding complex, whereas the slower migrating bands are referred to as the U/A-binding complex. Gel-shifted bands were not seen when the probes were incubated without extract (lane 7).
### Binding of RNP Complexes to the NF-L mRNA

*Fig. 2.* Gel shift assay of RNP complexes that bind to probe A680 (+1482/+2161) when incubated with cytosolic brain extract (120 μg) in the presence of 0 (−), 20 (+), 200 (+), or 2000 (++) ng of poly(U), poly(A), poly(C), or poly(G) homoribopolymers. A set of slower migrating bands (open arrowheads) are competed with poly(U) or poly(A) and are referred to as the U/A-binding complex.

A set of faster migrating bands (closed arrowheads) are competed with poly(C) and are referred to as the C-binding complex. Gel-shifted bands do not occur in the absence of extract (lane 1) or when the extract is treated with protease K (lane 2).

1) or when the extract was treated with protease K (lane 2) or heated to 65 °C (data not shown).

### C-binding Complexes Require the Proximal 45 nt of 3′-UTR and Distal 23 nt of 3′-CR for Binding

To localize binding sites, gel shift assays were conducted with probes that extended to varying distances into the 3′-UTR (Fig. 3). The C-binding complex (solid arrowheads) formed on the full-length A680 probe extending to +2161 (lanes 1–3), to a much lesser extent on probe A680/H extending to +1779 (lanes 4–8), but not on probe A680/B extending to +1712 (lanes 9–12) or on probe A680(del) in which sequence between +1712 and +1779 is deleted (lanes 13–15). Variations in relative intensities of gel-shifted bands of the U/A- and C-binding complexes occurred when examined with different probes. In addition, the C-binding complex was markedly enhanced on probe A680/H when U/A-binding complex was competed off with poly(U) or poly(A) (compare lanes 4 with lanes 6–8). The findings indicate that formation of the C-binding complex requires sequence between +1712 and +1779. This sequence comprises the distal 23 nt of 3′-CR (+1712/+1734) and the proximal 45 nt of 3′-UTR (+1735/+1779).

To confirm the location of the binding site for the C-binding complex, gel shift studies were conducted with probe A (+1712/+1779), which contained the 3′-CR and 3′-UTR components of the putative binding site, and with probe X (+1735/+1779), which contained only the 3′-UTR component of the binding site. Fig. 4 shows that the C-binding complex (solid arrowhead) readily formed on probe A (lane 1), but not very well on probe X (lane 6). Upon prolonged exposures of autoradiograms, similar but markedly reduced amounts of C-binding complex can be identified on probe X (data not shown). Low (20 ng), medium (200 ng), and high (2000 ng) amounts of poly(C) competed the C-binding complex from probe A (lanes 2–4) and probe X (data not shown). Note that the addition of poly(C) not only abolished the C-binding complex but also enhanced the formation of the slow-migrating U/A-binding complex (open arrowhead) on probe A (lanes 2–4) and probe X (data not shown). Additional gel-shifted bands formed on probe A and, especially, on probe X, but were not competed with poly(C) or poly(U), and their relationship to the C- and U/A-binding complexes is unclear. Their presence does not obscure the principle finding, i.e. that the C-binding complex binds weakly to the proximal 45 nt of 3′-UTR and that this binding is markedly enhanced by the addition of upstream flanking sequence.

Further evidence that 3′-CR is instrumental in the formation of the C-binding complex is seen in gel shift assays using a series of 5′-deletion probes extending from +2064 (probe 1), +1870 (probe 2), +1768 (probe 3), +1735 (probe 4), and +1519 (probe 5) to +2161 (see Fig. 1). Whereas probes 1–4 contained increasing amounts of 3′-UTR, only probe 5 extended beyond the 3′-UTR and into the 3′-CR. Fig. 5 shows that addition of poly(C) abolished the set of fast-migrating gel-shifted bands (solid arrowheads) that formed on probe 5 (compare lanes 1 and 3) but did not abolish the set of fast-migrating gel-shifted bands that formed on probe 4 (compare lanes 4 and 6), probe 3 (compare lanes 7 and 9), probe 2 (compare lanes 10 and 12) or probe 1 (compare lanes 13 and 15). The ability to be competed away with poly(C) identifies the fast-migrating set of bands on probe 5 as the C-binding complex. This complex was also enhanced in the presence of poly(U) (compare lanes 1 and 2). Gel-shifted bands of similar migration also formed on probes 1–4, but were not competed with poly(C), so that their relationship to the C-binding complex is unclear. The findings provide additional evidence that formation of the C-binding complex is dependent upon sequences in the proximal 3′-UTR and distal 3′-CR.

### U/A-binding Complexes Bind at Multiple Sites in 3′-UTR of NF-L mRNA

U/A-binding complexes are defined as sets of slow-migrating gel-shifted bands that are competed with

![Image](54x491 to 292x729)
poly(U) or poly(A). Fig. 5 shows that similar U/A-binding complexes (open arrowheads) were competed with poly(U) on probe 5 (lanes 1 and 2), probe 4 (lanes 4 and 5), probe 3 (lanes 7 and 8), probe 2 (lanes 10 and 11), and probe 1 (lanes 13 and 14). Gel-shifted bands of U/A-binding complexes on probes 1–5 differed in their relative abundance. Likewise, similar gel-shifted bands in different proportions comprised the U/A-binding complex that formed on probes containing only 3'9-CR sequence (A680/B), with the addition of proximal 3'-UTR (A680/H) or with the addition of the full 3'-UTR (A680) (see Fig. 3). U/A-binding complexes were also present on short probes (see Fig. 4) and were generally composed of fewer and fainter bands. Moreover, the U/A complexes were sometimes competed more effectively with poly(U) or poly(A), depending on the size and position of the probe. Finally, in many instances, competing off the U/A-binding complex with poly(U) or poly(A) enhanced the formation of the C-binding complex. We conclude that slow-migrating U/A-binding complexes of varying composition form at multiple sites in the 3'-CR and 3'-UTR of NF-L mRNA and that formation of U/A-binding complexes may adversely affect the formation of C-binding complexes.

Formation of C-binding Complexes Requires Multiple C-rich Sequences in the Proximal 3'-UTR—The sequence necessary for the formation of the C-binding complex extends from 1'1712 to 1'1779 of the NF-L cDNA and spans the distal 23 nt of 3'-CR and proximal 45 nt of 3'-UTR, as follows.

To assess specific sequence requirements for the formation of the C-binding complex, gel shift assays were conducted with brain extracts and probe A (1'1712/1'1779) that had been preincubated with 15-mer antisense oligonucleotides (100 ng) to the proximal (P), middle (M), or distal (D) sequence of the putative C-binding site and then incubated with brain extract in the presence of 0 (–) or 2000 (+++) ng of poly(C) or poly(U). Control 15-mer antisense oligonucleotides were from the SK+ vector (SK) and from the 5'-flanking region of NF-L (5').
affect the formation of C-binding complexes. The findings indicate that assembly of the C-binding complexes can be disrupted at multiple sites, thereby requiring an extended stretch of intact sequence in the proximal 3′-UTR that is enriched in cytosine and pyrimidine residues.

Components for Assembly of C-binding Complexes Are Present in Cytosolic Extracts from Neuronal and Non-neuronal Cell Lines but Are Enriched in Neuronal Tissues—Cytosolic extracts from neuronal (P19 and N2a) and non-neuronal (L cells) cell lines were tested in gel shift assays and were found to generate C-binding complexes (data not shown). Studies were then conducted to compare the C-binding complexes from neuronal (brain) and non-neuronal (liver) tissues. Fig. 7 shows that very similar C-binding complexes formed when brain (lanes 3–8) or liver (lanes 9–14) extracts were gel-shifted with probe A. However, at least a 10-fold larger amount of C-binding complex formed with brain extracts, based upon radioactivity per microgram of protein in the cytosolic extract.

C-binding Complexes Contain 18- and 36-kDa Polypeptides, whereas the U/A-binding Complexes Contain 72- and 80-kDa Polypeptides—Components of the C-binding and U/A-binding complexes were identified by UV cross-linking of the complexes that form on radioactive probes, then digested away the radioactive residues that are not cross-linked to protein and using the cross-linked radioactivity to detect polypeptides in autoradiograms after their separation by SDS-polyacrylamide gel electrophoresis. When polypeptides in rat brain extracts were cross-linked to probe A (+1712/+1779) and separated on a 10% gel (Fig. 8B), 80- and 72-kDa polypeptides were competed away by the addition of poly(U) (compare lanes 2 and 4) and were slightly increased by the addition of poly(C) (compare lanes 2 and 3). A 39-kDa polypeptide was competed away by the addition of poly(C) (compare lanes 2 and 3) and was slightly increased by the addition of poly(U) (compare lanes 2 and 4). The presence of an 18-kDa polypeptide (lane 2) and its ability to be competed by poly(C) (lane 3) and enhanced by poly(U) (lane 4) were obscured by the cross-linking of additional polypeptides that migrated in the 20–30-kDa range (compare lanes 1 and 2). The findings identify 18- and 36-kDa core polypeptide components of the C-binding complex and 80- and 72-kDa components of the U/A-binding complex.

**DISCUSSION**

With this study, we have begun to identify the RNP binding factors and binding sites that regulate the stability of NF-L mRNA. Competition with homoribopolymers has been very helpful in characterizing specific RNP components and in localizing their binding sites. In particular, the ability to be selectively competed with poly(C) has identified a prominent C-binding complex that binds to the proximal 3′-UTR of NF-L mRNA. In a companion study, we have mapped the stability determinants in the NF-L transcript and have shown that the major determinant of NF-L mRNA stability is localized to the binding site of the C-binding complex. The identification and characterization of RNP complexes on the NF-L transcript are therefore directly relevant to the mechanisms that regulate the stability of the NF-L transcript.

The C-binding complex is unusual, in that its binding site is located in the proximal 45 nt of 3′-UTR but the binding reaction is markedly enhanced by the addition of the 23-nt flanking upstream sequence in the 3′-CR. The ability of distal 3′-CR to enhance formation of C-binding complexes could be due to its participation in secondary (or tertiary) structure. Computer modeling based on the free energy minimization algorithm of Zuker et al. (19) indicates that the distal 3′-CR forms a stable stem structure with the proximal 3′-UTR. Alternatively, it is also possible that other factors bind to the 3′-CR and facilitate formation of the C-binding complex. Immediate upstream flanking sequences are necessary for binding to a 29-nt destas-
bilizing element in the 3′-UTR of human amyloid precursor protein mRNA (20). Remote upstream sequences may also be required to form an essential stem structure (21).

The binding site for the C-binding complexes in the proximal 3′-UTR of NF-L mRNA is phylogenetically conserved (Table I). Several pyrimidine-rich stretches are present in proximal 45 nt of the 3′-UTR and may be instrumental for binding of the complex. Disruption of RNA structure by incubating probes with antisense oligonucleotides shows that the C-binding complex requires the integrity of sequences spanning the proximal, middle and distal pyrimidine-rich sites. Flanking and intervening sequences are also conserved (Table I) and may also be important for the formation of the C-binding complex.

Pyrimidine-rich stretches in the proximal 3′-UTR are also assembly sites for RNP complexes that regulate stability of other mRNAs (16, 17, 22–24). The pyrimidine composition of some of these sites is C-rich and complex formation is also inhibited by poly(C) homoribopolymers (16, 22). Moreover, pyrimidine-rich sites often contain tandem repeats of sequence-specific motifs that are not functionally redundant in that complex formation is markedly impaired if a single motif repeat is disrupted (16, 22, 24). Whereas point mutations in some residues can be very disruptive, complex formation may tolerate or even be enhanced by exchanging the C and U pyrimidine residues in the binding site (22, 25). Whereas point mutations in some residues can be very disruptive, complex formation may tolerate or even be enhanced by exchanging the C and U pyrimidine residues in the binding site (22, 25). Interestingly, the sequence as well as the recognition site in the cognate binding factors have diverged during evolution so that the complex on the α-globin mRNA binds to a C-rich motif in the human but C/U-rich motif in the mouse (17). Splicing factors that bind to polypyrimidine tracts of intervening sequences have distinctive but overlapping sequence specificities (26–28). Indeed, competition between multiple trans-acting factors for binding to polypyrimidinic tracts is believed to be instrumental in splicesome assembly and in the selection of the 3′- splice sites.

An unusual feature of the C-binding complex is that its assembly is effected by the presence of other RNP complexes on the transcript. Gel shift studies suggest that the C- and U/A-binding complexes have reciprocal binding interactions, as if the two sets of complexes compete for the same components or use the same or nearby sites. These binding features were most readily observed with short probes that spanned the C-binding and nearby U/A-binding sites. Moreover, reciprocal binding phenomena were readily apparent in cross-linking experiments, suggesting that interactions between the complexes alter core (i.e. RNA contacting) binding components of the respective complexes.

The assembly of similar U/A-binding complexes on probes to different regions (e.g. probes A, 1, and A680/B) of the NF-L transcript indicates that the complex binds to multiple sites in the 3′-UTR and 3′-CR. Such a multiplicity of binding sites in the 3′-UTR and 3′-CR is reminiscent of some adenylate/uridylate-rich elements (ARE) that were originally described as destabilizing determinants in short-lived mRNAs of proto-oncogene, cytokines, and transcription factors (32). For example, destabilization of β-interferon mRNA is mediated by multiple AREs that compete with each other and with poly(U) or poly(A)
in the binding of a 65-kDa polypeptide (33). Agonist- or hypoxia-induced stabilization of mRNAs may be mediated by masking destabilizing AREs (34) or by a different set of AREs (35). Binding of the same set of factors to multiple sites in the 3′-UTR is also believed to regulate the stability of the neuronal GAP-43 mRNA (31). Moreover, the GAP-43 binding factors are neuron-specific, including a factor that was recently identified as an Elav-like protein (36). Interestingly, Elav-like proteins are essential for the differentiation and maintenance of neurons and have been identified as the target immunogens that mediate autoimmune neurodegenerative disease (37).

The extent to which different components of the C- and U/A-binding complexes are neuron-specific is presently unknown. It is quite possible, for example, for neuron specificity to be mediated by altering (e.g. phosphorylation) a common component, by changing the concentration of a critical component(s), or by adding a novel component to the RNP complex in a neuronal setting. The 18- and 36-kDa polypeptide core binding components of the C-binding complexes are novel RNP components, which are enriched in neuronal tissues. They could represent subunits with similar properties or monomer/dimer components of a common subunit. In the latter instance, the ability of poly(C) to compete preferentially with the binding of the 36-kDa polypeptide would indicate a greater effect on the assembly of the dimer in the complex. In either case, the subunits are smaller than those of other RNP components that bind to C-rich sites and are competed with poly(C) (16, 22, 29–31).

The role of the C-binding and U/A-binding complexes in regulating the stability of the NF-L mRNA is presently unknown. The identification of the complexes and their binding sites will now enable further characterization of the RNP components and their role in stabilizing NF-L transcript. It is quite conceivable that factors regulating NF-L mRNA stability could also play a major role in the post-transcriptional regulation of neuronal metabolism. In particular, the findings could provide important insights into the relationship between overexpression of an NF-L transgene and the selective degeneration of motor neurons in transgenic mice.

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