

Neurofilaments Are Obligate Heteropolymers In Vivo

Michael K. Lee,* Zuoshang Xu,* Philip C. Wong,* and Don W. Cleveland*‡

Departments of *Biological Chemistry and ‡Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. Neurofilaments (NFs), composed of three distinct subunits NF-L, NF-M, and NF-H, are neuron-specific intermediate filaments present in most mature neurons. Using DNA transfection and mice expressing NF transgenes, we find that despite the ability of NF-L alone to assemble into short filaments in vitro NF-L cannot form filament arrays in vivo after expression either in cultured cells or in transgenic oligodendrocytes that otherwise do not contain a cytoplasmic intermediate filament (IF) array. Instead, NF-L aggregates into punctate or sheet like structures. Similar nonfilamentous structures are also formed when NF-M or NF-H is expressed alone. The competence of NF-L

to assemble into filaments is fully restored by coexpression of NF-M or NF-H to a level ~10% of that of NF-L. Deletion of the head or tail domain of NF-M or substitution of the NF-H tail onto an NF-L subunit reveals that restoration of in vivo NF-L assembly competence requires an interaction provided by the NF-M or NF-H head domains. We conclude that, contrary to the expectation drawn from earlier in vitro assembly studies, NF-L is not sufficient to assemble an extended filament network in an in vivo context and that neurofilaments are obligate heteropolymers requiring NF-L and NF-M or NF-H.

IN most eukaryotic cells, the cytoskeleton is composed of three distinct structural components with characteristic diameters: actin-microfilaments (7 nm), intermediate filaments (IFs)¹ (8–10 nm), and microtubules (24 nm). The subunits from which cytoplasmic and nuclear IFs are assembled have been categorized into five distinct groups based on primary amino acid sequence differences and the intron positions of the genes encoding the subunits (Steinert and Roop, 1988). Types I and II include acidic and basic keratins expressed primarily by epithelial cells. The type III consists of vimentin expressed in cells of mesenchymal origin, desmin expressed in muscle cells, GFAP expressed in astrocytes, and peripherin expressed by a subset of neurons in the peripheral nervous system. Type IV consists of neurofilaments (NFs) expressed by most mature neurons, although nestin, expressed in neural precursor cells and α -internexin, expressed in subset of neurons particularly during early neurite outgrowth, probably belong to type IV as well (Fliegner et al., 1990; Cleveland et al., 1991). The nuclear lamins that form the filaments underlying the nuclear membrane constitute type V. All of these IF proteins are characterized by an α -helical domain of ~310 (or 352 for the lamins) amino acids that is flanked by globular amino-terminal head and carboxyl-terminal tail domains whose lengths vary and whose amino acid sequences are not conserved.

In most mature neurons, particularly in the neurons that give rise to large myelinated axons, the IF system is almost exclusively composed of neurofilaments. In these axons, neurofilaments are by far the most abundant cytoskeletal element, often exceeding the number of microtubules by an order of magnitude. Several lines of evidence have collectively demonstrated that proper assembly and organization of neurofilaments in axons are important for specifying the axonal diameter of the large caliber myelinated axons (see Cleveland et al., 1991; Yamasaki et al., 1991). More recently, transgenic mice models have shown that disruption of normal neurofilament subunit content by over-expression of either NF-L or NF-H results in abnormal assembly and aggregation of neurofilaments in neuronal cell bodies, particularly in the motor neurons (Côté et al., 1993; Xu et al., 1993). In these transgenic mice, the disruption of normal organization of neurofilaments was accompanied by motor neuron defects that ultimately resulted in denervation-induced atrophy of skeletal muscles. The role of neurofilaments in these pathogenic events highlights the importance of identification and characterization of domains in NF subunits that participate in normal cytoplasmic assembly and organization.

Unlike most other non-epithelial IFs, which are homopolymers (e.g., vimentin, desmin, and GFAP), neurofilaments in mature neurons are assembled from three separate subunits called NF-L, NF-M, and NF-H, with true molecular masses of ~60 kD (NF-L), ~100 kD (NF-M), and ~120 kD (NF-H) (reviewed by Nixon and Shea, 1992). The presence of a conserved 310 amino acid α -helical rod domain on

1. *Abbreviations used in this paper:* CMV, Cytomegalovirus; GFAP, glial fibrillary acidic protein; IF, intermediate filament; MSB, microtubule stabilization buffer; MSV, murine sarcoma virus; NF, neurofilament.

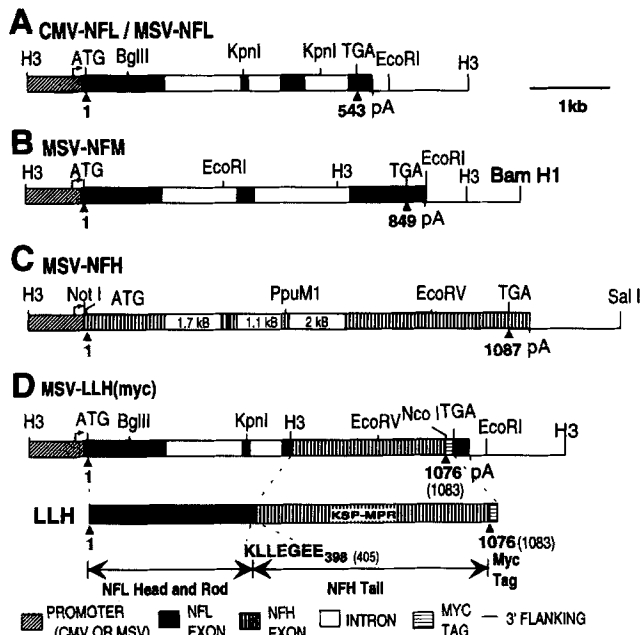


Figure 1. Gene constructs for expression of NF-L, NF-M, NF-H, and LLH-hybrid subunits. (A) Schematic drawing of CMV-NFL or pMSV-NFL genes. Schematic drawing of (B) MSV-NFM gene; (C) MSV-NFH gene; and (D) MSV-LLH gene. The introns in C are not drawn to scale and the actual size of introns are indicated. (D) Shown at the bottom of D is a schematic drawing of MSV-LLH polypeptide. The encoded amino acid numbers are shown below the arrowheads. The numbers in the parentheses represent the amino acid numbering from the wild-type NF-H polypeptide for the NF-H tail segment in LLH.

all three NF subunits indicates that each may be an integral subunit of the 10-nm NFs. Assembly studies using purified NF-subunits show that only NF-L and NF-M, but not NF-H, are able *in vitro* to form 10-nm filaments (Gardner et al., 1984; Geisler and Weber, 1981; Liem and Hutchinson, 1982), although even under optimal conditions NF-M self-assembles into short, irregular filaments and only under very limited buffer conditions. Both NF-M and NF-H can be incorporated into filaments in the presence of NF-L, indicating that determinants for coassembly are contained within the individual subunits (Balin and Lee, 1991; Hisanaga and Hirokawa, 1988; Zackroff et al., 1982). These *in vitro* assembly experiments, along with antibody decoration of native neurofilaments (Hirokawa et al., 1984; Willard and Simon, 1981), have led to the conclusion that *in vivo* neurofilaments are probably assembled from an NF-L core and that NF-L is necessary and sufficient to form filaments. Thus, potential functional roles for both NF-M and NF-H have focused on interactions mediated by the long carboxyl-terminal tail domains (438 and 676 amino acids for NF-M and NF-H, respectively) which are targets for multiple phosphorylation events (see Nixon and Shea, 1992). However, interpretation of neurofilament reassembly studies is not as straightforward as it may seem. *In vitro* reassembly of neurofilaments requires acidic pH, is inefficient when compared to the reassembly of either Type III filaments (vimentin) or Type I/II filaments (keratin) and yields short, somewhat irregular filaments. Moreover, neurofilaments reassembled

in vitro do not approximate the parallel arrays of organized neurofilaments seen in all regions of a neuron.

In vivo assembly characteristics of NF subunits have also been studied by expressing NF subunits in fibroblast cells in culture and examining the coassembly of NF subunits with endogenous vimentin filaments (Monteiro and Cleveland, 1990; Gill et al., 1990; Wong et al., 1990; Chin and Liem, 1990; Chin et al., 1991). Using such pseudo-*in vivo* systems, the rod as well as portions of the head and tail domains of both NF-L and NF-M have been shown to be important for coassembly with vimentin into a cytoplasmic network of filaments. We have now extended such analyses by examining the self-assembly and organization of NF subunits into a cytoplasmic neurofilament network in cells that do not express any cytoplasmic intermediate filaments. What we find is that the *de novo* assembly of neurofilaments *in vivo* requires both NF-L and either NF-M or NF-H. Further, we demonstrate that the amino-terminal head, but not the carboxyl-terminal tail, of NF subunits are required for assembly of a neurofilament network *in vivo*.

Materials and Methods

Construction of Hybrid NF Genes

The construction of murine sarcoma virus (MSV) promoted NF genes, as well as amino and carboxyl-terminal truncation mutants, have been described (Monteiro and Cleveland, 1990; Gill et al., 1990; Wong and Cleveland, 1990). Cytomegalovirus promoted vimentin (pCMV-vimentin) (Somers et al., 1992) was a kind gift from Dr. Connie Sommers (National Institutes of Health, Bethesda, MD).

To construct pCMV-NFL (Fig. 1 A), pNF-L-Bam (Monteiro and Cleveland, 1990) was linearized at the BamHI site located between the promoter and the ATG translation initiation codon and blunted with Klenow and dNTP. The DNA was then digested with HindIII and the 5-kb NF-L gene fragment missing the 5'-promoter region was isolated. To produce pCMV, the 600-bp human CMV early promoter was cloned between KpnI and XhoI sites in pGEM-3z. To produce pCMV-NFL, pCMV was digested with XhoI, blunted with Klenow and digested with HindIII. The 5-kb 5'-blunt/3'-HindIII NF-L fragment from above was then ligated into blunt/HindIII sites 3' to the CMV promoter.

To construct pMSV-NFH (Fig. 1 C), a 12-kb fragment containing the mouse NF-H gene was obtained by double digesting Cos3A1 (kindly provided by Dr. J.-P. Julien, Montreal, Canada; Julien et al., 1988) with KpnI and SalI. To obtain pNFH, the 12-kb NF-H gene was cloned between KpnI and SalI sites of pUC19. To obtain pMSV-NFH, the NF-H promoter in pNFH was removed using KpnI and NotI and replaced with the MSV promoter described below. A 600 base (HindIII-BglIII fragment) containing the MSV promoter was subcloned between the HindIII-BamHI sites of pBluescriptII-KS⁺ (pBSII-KS⁺). The MSV promoter was then excised with KpnI and NotI and ligated in place of NF-H promoter.

To construct pMSV-LLH (Fig. 1 D), oligonucleotide-directed mutagenesis was used to create HindIII restriction sites in both NF-L and NF-H genes and at exactly the same position in the carboxy-terminal portion of the rod, which ends in a sequence (RKLEGE) common to both NF-L and NF-H. The nucleotide changes did not alter the amino acid sequence (Fig. 1 D). For NF-H, a 2.3-kb EcoRI fragment (encoding amino acids 267-1053 of NF-H) from a partial NF-H cDNA clone, pMuHI (kindly provided by Dr. P. Shneidman, University of Pennsylvania; Shneidman et al., 1988), was subcloned into the EcoRI site of M13mp18. Single-stranded DNA was obtained from a clone with desired orientation and a HindIII site was engineered into the gene by oligonucleotide-directed mutagenesis using the Amersham kit, version 2.1 (Amersham Corp., Arlington Heights, IL). The mutants were confirmed by DNA sequencing. Double-stranded DNA was recovered and digested with HindIII and EcoRV. The mutated 1.3-kb fragment was cloned between the EcoRV-HindIII sites of pBSII-KS⁺ to produce pMuHI(H3)-BS. To introduce a HindIII site in the NF-L gene, 1 kb KpnI fragment containing the sequences encoding the terminal rod domain (including the KLEGE) was subcloned into the KpnI site of pBSII-KS⁺. Single stranded DNA templates were rescued (using helper phage R408)

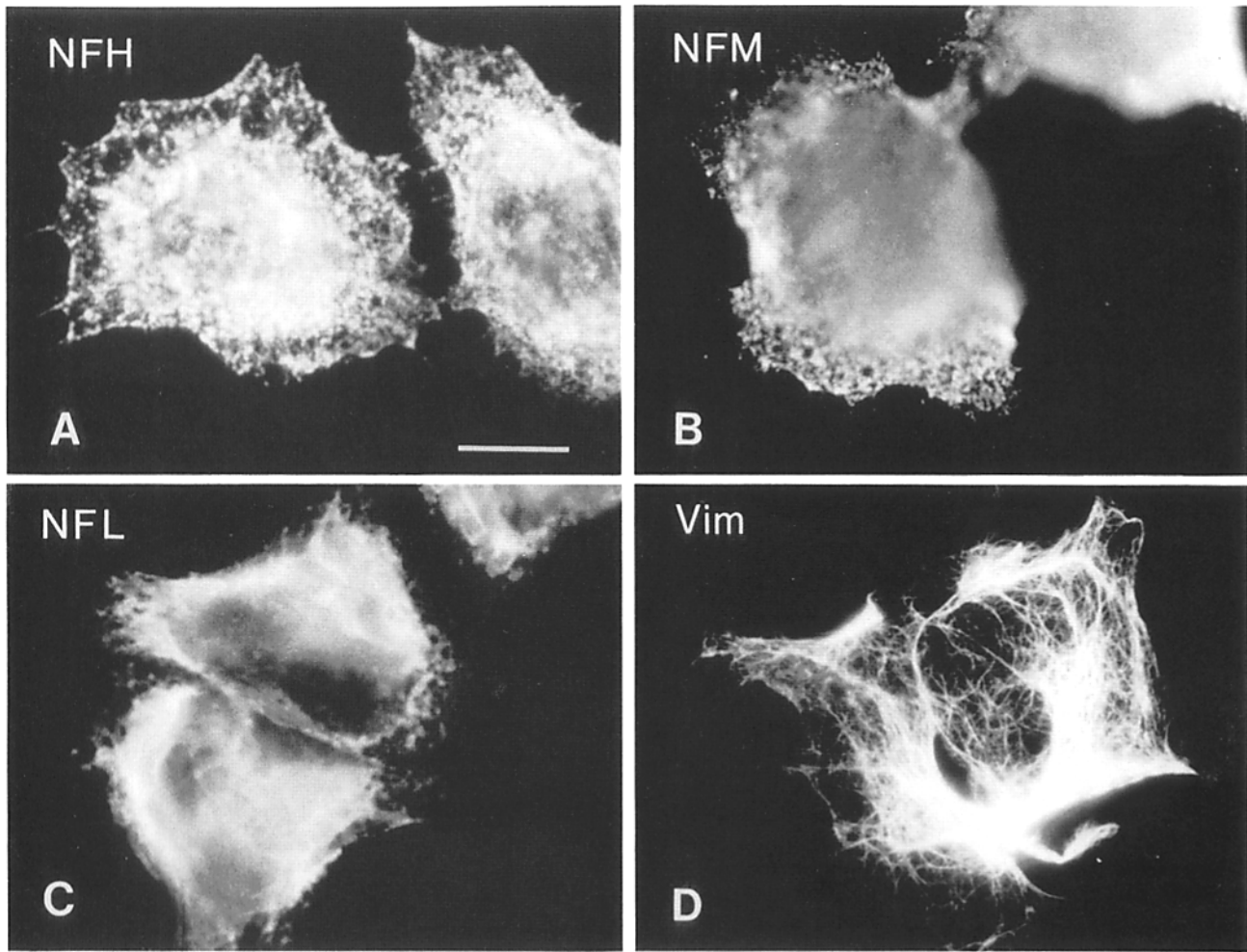


Figure 2. Individual NF subunits are self-assembly incompetent in an *in vivo* context. IF⁻ SW13 cells were grown on glass coverslips and transfected with either (A) pMSV-NFH, (B) pMSV-NFM, (C) pMSV-NFL, or (D) pCMV-Vim. Using immunofluorescence microscopy, (A) NF-H and (C) NF-L were localized by affinity-purified rabbit polyclonal antibodies to either NF-L or NF-H followed by fluorescein-conjugated goat anti-rabbit IgG. (B) NF-M and (D) vimentin were localized by mouse anti-NF-M or anti-vimentin mAbs followed by fluorescein-conjugated horse anti-mouse IgG. Bar, 10 μ m.

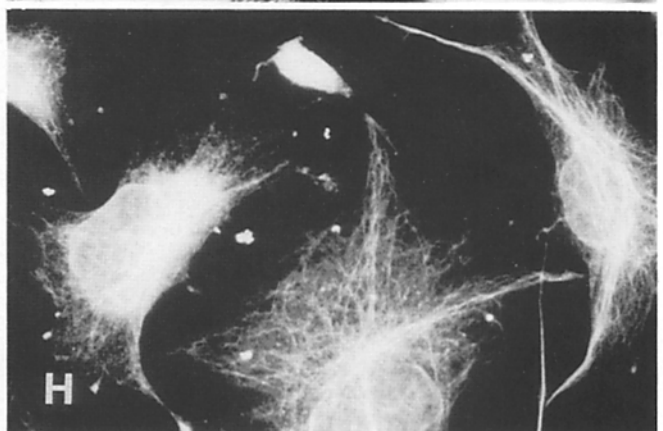
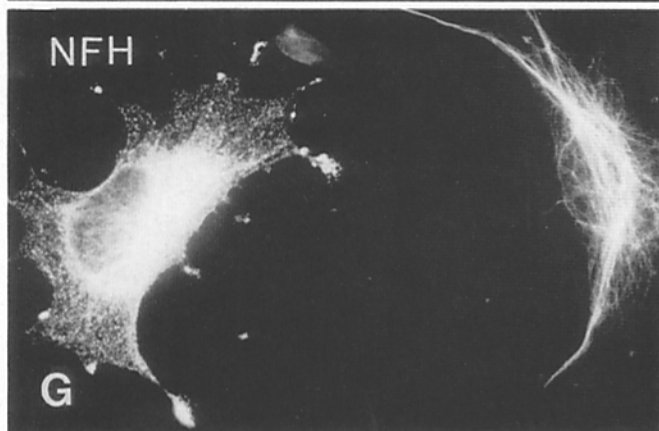
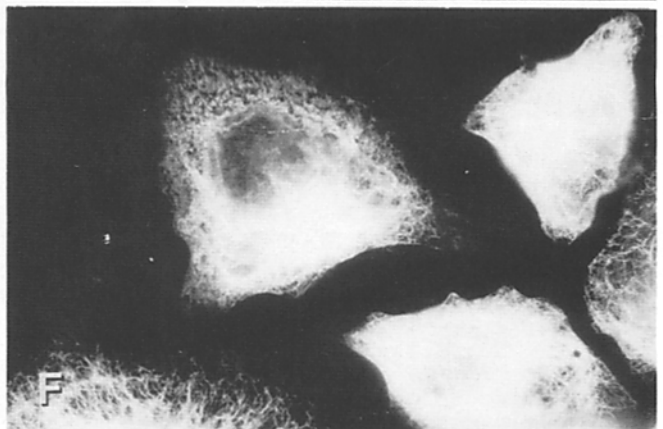
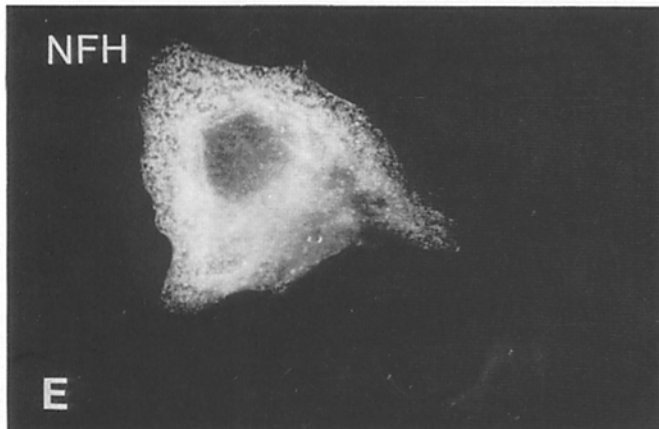
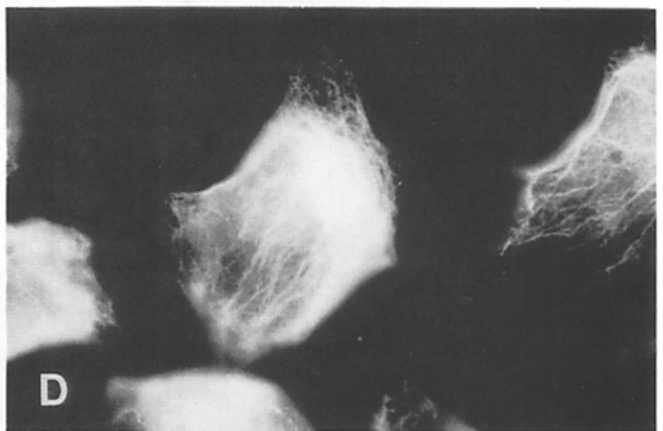
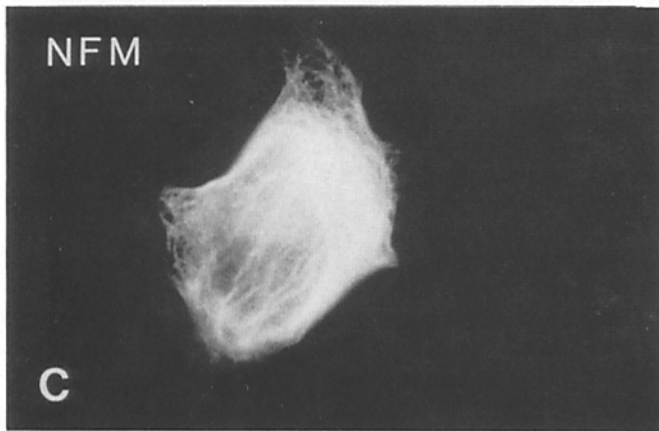
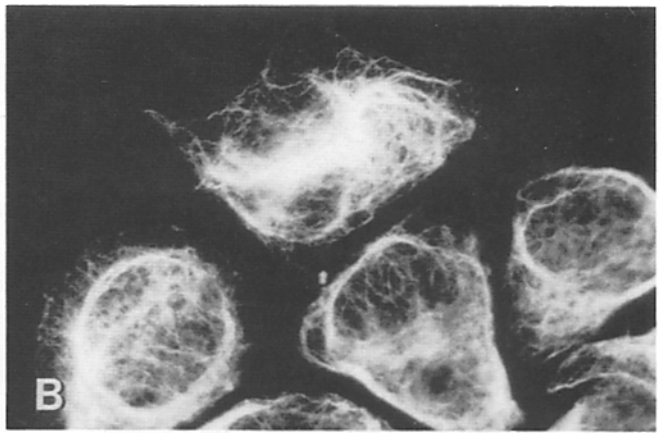
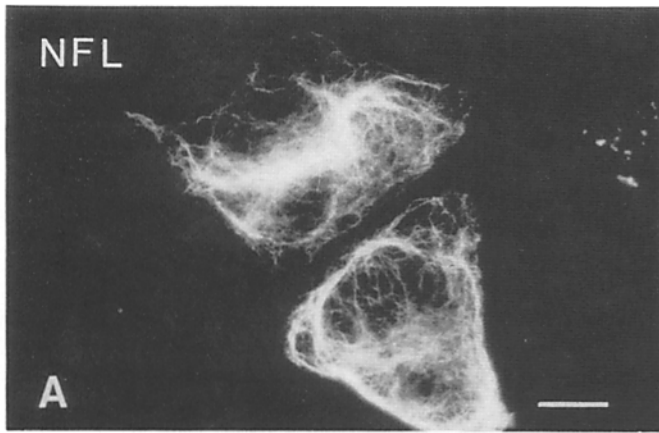
from a clone with the desired insert orientation and a HindIII site was introduced by oligonucleotide-directed mutagenesis. The desired clone, pNFL(Kpn-H3), was identified using both restriction digestion with HindIII and DNA sequencing. pNFL(Kpn-H3) was then digested with KpnI and HindIII to obtain a 450-bp fragment containing the sequences just 5' to the KLEGE-domain of NF-L. This fragment was ligated 5' to pMuHI(H3)-BS, which had been linearized by double digesting with KpnI and HindIII. The resulting clone, pL(Kpn)HI(H3)-BS, contains the 3'-half of NF-L exon 2, intron 3 and the engineered HindIII site located 1 base into exon 3, followed by the NF-H tail region up to sequences encoding amino acid 798. To complete the carboxyl-terminal region of NF-H and to add a *myc*-epitope tag, an 870 base fragment beginning at codon 799 and ending at the normal translation termination codon was obtained by PCR. The PCR primers were designed so that the last four amino acids and the termination codon of NF-H were replaced with a NcoI site followed by a 12-amino acid *myc*-tag. The PCR fragment was digested with NcoI and ligated into *pmyc*-0 (a plasmid carrying the *myc*-tag followed by the NF-L 3' gene flanking sequences; Wong and Cleveland, 1990) that had been first digested with SalI, blunted by Klenow and digested with NcoI. The resulting clone (pNFHc284-*myc*) contains the NF-H sequences encoding the last 284 amino acids of NF-H tail followed by the *pmyc*-0 sequences. From pNFHc284-*myc*, a 1.4-kb fragment encoding 284 amino acids of NF-H, the *myc*-tag and the NF-L poly A-signal was excised by double digesting with EcoRV and EcoRI. To obtain pH(Kpn-H3)Ht, this 1.4-kb fragment was ligated to pL(Kpn)HI(H3)-BS that had been linearized by double digesting with EcoRV and EcoRI.

pMSV-LLH was built from three parts. To obtain the first of these, pMSV-NFL (Monteiro and Cleveland, 1990) was double digested with BglII and EcoRI, yielding the pUC vector plus the MSV promoter, NF-L sequences in exon I up to a BglII-site, and the NF-L 3'-flanking region. For the second part, the NF-L gene was double digested with BglII and KpnI to obtain a 1.8-kb fragment containing the 3'-end of NF-L exon 1, NF-L intron 1 and 5'-half of exon 2. For the third fragment, pL(Kpn-H3)Ht was double digested with KpnI and EcoRI; the resulting 3-kb fragment contained the NF-sequences and the *myc*-tag was purified. These fragments were ligated together to obtain pMSV-LLH.

Tissue Culture and Transfection

Vimentin containing (cl.1; IF⁺) and vimentin negative (cl.2; IF⁻) clones of the SW13 cell line (derived from a human adrenal carcinoma (Sarría et al., 1990) were kindly provided by Dr. Robert M. Evans (Department of Pathology, University of Colorado Health Sciences Center). The SW13 cells were grown in DME supplemented with 10% FBS. The SW13 cells were transiently transfected using the calcium phosphate co-precipitation procedure (Graham and van der Eb, 1973). The cells were routinely analyzed 40 h after transfection.

Mouse fibroblast L-cells were maintained in DME supplemented with 10% FBS. The L-cells were transiently transfected using the DEAE-dextran method (Lopata et al., 1984). Cells were analyzed 40 h after transfection.



Immunofluorescent Staining and Light Microscopy

Cells grown on glass coverslips were washed in microtubule stabilization buffer (MSB: 4 M Glycerol, 100 mM Pipes-KOH, pH 6.9, 1 mM EGTA and 1 mM MgSO₄) for 30 s at 35°C, extracted for 60 s in MSB containing 0.4% (wt/vol) Triton X-100 at 35°C and briefly rinsed in MSB. The samples were then fixed by immersion in -20°C methanol for 10 min. The samples were rehydrated in PBS, blocked with 2% nonfat dry milk for 15 min at room temperature, washed in PBS and incubated with a primary antibody (diluted in PBS containing 0.2% nonfat dry milk) for 1-2 h at room temperature in a humidified chamber. After the primary antibody incubation, coverslips were washed three times in PBS, incubated for 1 h in secondary antibody, washed again in PBS, and mounted on glass slides using Aquamount (Lerner Laboratories, New Haven, CT). The cells were examined on an Olympus BH-2 microscope using epifluorescence optics and photographed on Kodak T-MAX film (Eastman Kodak Co., Rochester, NY). Proteins containing the 12-amino acid *myc* epitope tag were detected using either an anti-*myc* mouse mAb (*myc1-9E10*; Evan et al., 1985) or affinity-purified rabbit polyclonal antibodies generated against a *myc* synthetic peptide (CYG-MEQLISEEDLN). Specific NF subunits were also detected using mouse mAbs to either NF-L, NF-M, or NF-H, and affinity-purified rabbit polyclonal antibodies to NF-L or NF-H (Xu et al., 1993). A goat anti-vimentin polyclonal antibody (ICN Immunobiologicals, Costa Mesa, CA) and an anti-vimentin mouse mAb (V8; Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN) were used to detect vimentin. Appropriate affinity-purified secondary antibodies conjugated to either Texas red or fluorescein were used.

SDS Gel Electrophoresis and Immunoblotting

Cells were usually harvested 40 h after transfection, washed once with PBS, once with MSB at 35°C, extracted for 1 min with MSB containing 0.4% Triton X-100 at 35°C, and washed once with MSB at 35°C. The remaining cytoskeletal fraction was solubilized in 2% (wt/vol) SDS in 50 mM Tris-HCl, pH 6.8. Extracts were boiled for 5 min (100°C) and protein concentrations were determined using the bicinchoninic acid method (Smith et al., 1985). Extracts were then diluted with SDS sample buffer containing β -mercaptoethanol, boiled again for 5 min, and equal amounts of cytoskeletal proteins were separated by electrophoresis on 7.5% polyacrylamide gels (Laemmli, 1970). The separated proteins were immunoblotted (Lopata and Cleveland, 1987) and NF-L, NF-M, NF-H and vimentin were detected by autoradiography using mouse monoclonal primary antibodies against the appropriate proteins and ¹²⁵I-labeled goat anti-mouse secondary antibody. To quantify the amount of NF subunits accumulated in any sample, a series of twofold dilutions of neurofilaments purified from mouse spinal cord and containing known amounts of each NF subunit were immunoblotted in parallel with the samples of interest. The immunoreactivity of specific NF-subunit bands was quantified by densitometric scanning of the autoradiogram and compared with a standard curve constructed from densitometric scan of the neurofilament standards.

Ultrastructural Analysis of Transgenic Mice

Characterization of transgenic mouse lines expressing wild-type NF-L under the control of a MSV promoter (MSV-NFL-58 and MSV-NFL-103) has been previously described (Xu et al., 1993; Monteiro et al., 1990). Similar transgenic animals expressing a NF-M polypeptide in which the last 50 carboxyl-terminal amino acids were replaced by a 12-amino acid *myc* epitope tag (MSV-NF-Mc Δ 50; Wong et al., 1990) were generated by standard transgenic methods. A detailed characterization of these mice will be described elsewhere (Wong, P. C., J. Marszalek, and D. W. Cleveland, manuscript in preparation). Transgenic mice expressing both NF-L and NF-Mc Δ 50 in oligodendrocytes were engineered by mating MSV-NFL-58 with mice expressing MSV-NF-Mc Δ 50.

Mouse tissues were fixed for immunocytochemistry by intracardial per-

fusion with either 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.6. For EM, tissues were fixed by intracardial perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate, pH 7.6. For immunocytochemistry, the spinal cords were cryoprotected in 20% glycerol in 0.1 M sodium phosphate, pH 7.6 and frozen sections were cut (40 μ m). The transgenic NF subunits in nonneuronal cells in the spinal cord white matter were localized using double immunofluorescence microscopy. NF-L and NF-Mc Δ 50 were colocalized using affinity-purified anti-NFL rabbit polyclonal antibodies (Xu et al., 1993) and anti-*myc* mouse mAb (*myc1-9E10*; Evan et al., 1985) as primary antibodies. Astrocytes were identified using a rabbit polyclonal antibody to GFAP (Dako Corp., Carpinteria, CA).

For EM, paraformaldehyde/glutaraldehyde fixed spinal cords were post-fixed with 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in alcohol and embedded in Epon-Araldite resin. Thin sections were cut and stained with uranyl acetate and lead citrate and examined in an Hitachi H-600 transmission electron microscope.

Results

Individual Neurofilament Subunits Are Self-assembly Incompetent In Vivo

To examine the ability of each NF subunit to self-assemble an extended, de novo filament network in vivo, wild-type mouse NF-L, NF-M, and NF-H were individually expressed in IF⁻ SW13 cells, a cloned subline in which vimentin expression has been spontaneously silenced (Sarria et al., 1990). NF subunits and vimentin were localized 40 h after transfection using double immunofluorescence microscopy. (Simultaneous localization of vimentin and NF subunits was routinely done to control for the small percentage of the cells [\sim 1-5%] that spontaneously revert to IF⁺ phenotype.) As expected from its known in vitro assembly properties, in the absence of an endogenous IF array, NF-H failed to assemble into a filament network and localized to nonfilamentous, punctate structures that extended out to the peripheral margins of the cell (Fig. 2A). When expressed alone, NF-M was also incompetent to self-assemble within an in vivo context and localized to nonfilamentous structures similar to those seen with NF-H (Fig. 2B). In view of its assembly competence in vitro, expression of NF-L in IF⁻ cells produced an even more surprising result. At lower levels of accumulation, NF-L was restricted to fine punctate structures (not shown) similar to those seen with NF-M and NF-H. At higher levels of NF-L (achieved with the CMV-NFL gene), NF-L formed large nonfilamentous aggregates that covered large portions of the cytoplasm (Fig. 2C).

The absence of an IF network assembled from individual neurofilament subunits does not represent an inherent defect in IF assembly in the SW13 IF⁻ line: forced expression of human vimentin yielded a well elaborated vimentin filament network in the same cells (Fig. 2D; also see Sarria et al., 1990). Quantitative immunoblotting to measure average levels of accumulated NF-L, NF-M, NF-H, or vimentin showed that failure to assemble networks from the NF subunits could not be explained by low levels of expression (see below).

Figure 3. Coassembly of individual NF subunits with endogenous vimentin in IF⁺ SW13 cells. SW13-cl.1 cells were grown on glass coverslips and transfected with plasmid (A and B) pCMV-NFL, (C and D) pMSV-NFM and (E and F) pMSV-NFH. Mouse L-cells grown on glass coverslips were transfected with (G and H) pMSV-NFH. (A, C, and G) NF subunits were localized using affinity-purified rabbit polyclonal antibodies against (A) NF-L or (E) NF-H or mouse mAbs against (C) NF-M and (G) NF-H. Appropriate fluorescein-conjugated secondary antibodies were used to visualize expression by indirect immunofluorescence microscopy. (B, D, F, and H) Vimentin in the same transfected cells was visualized using double immunofluorescence microscopy with (B and F) mouse mAbs or (D and H) goat polyclonal antibodies to vimentin followed by appropriate Texas red-conjugated secondary antibodies. Bar: (A-F) 10 μ m; (G and H) 16 μ m.

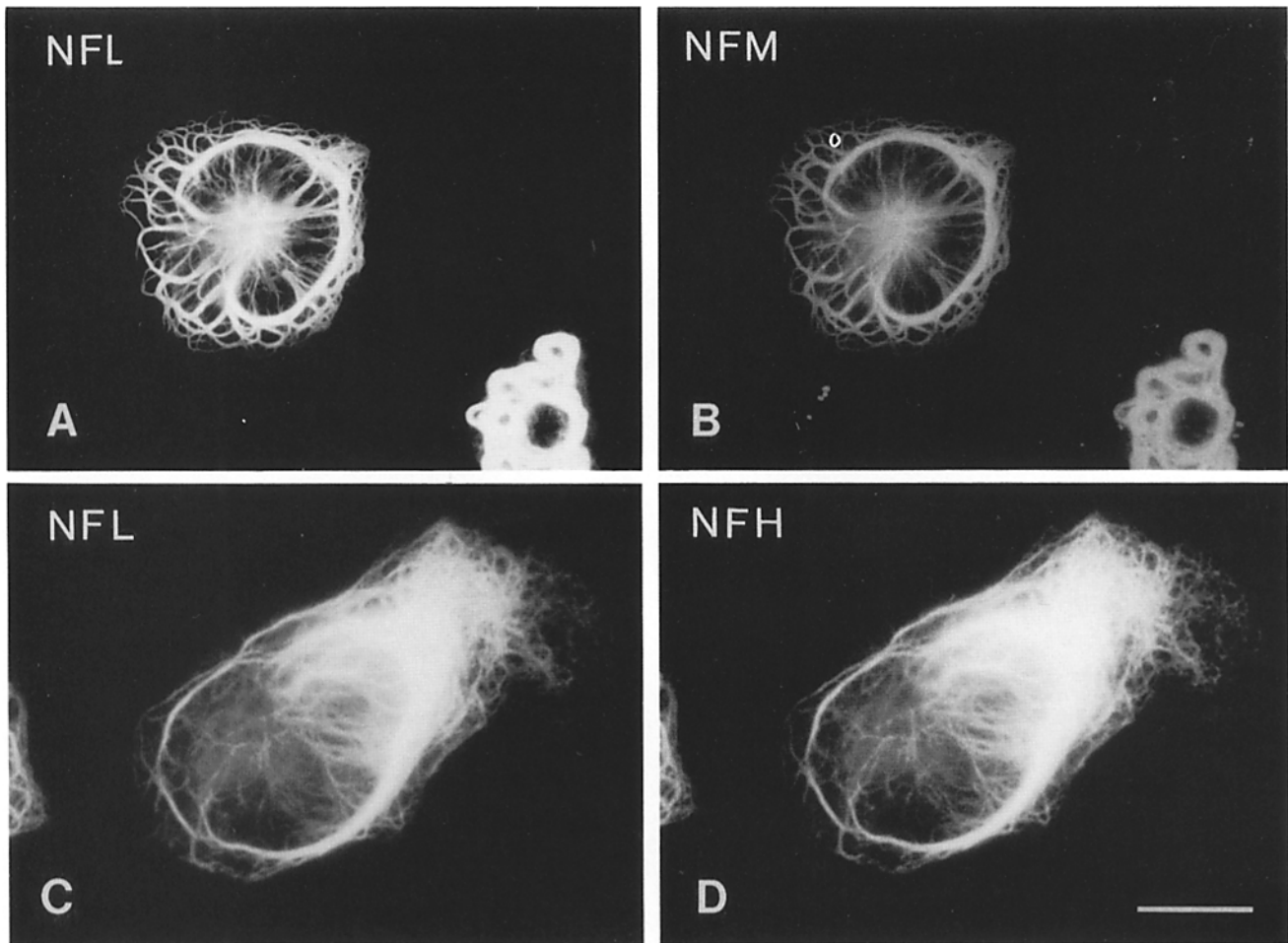


Figure 4. Coexpression of NF-L with either NF-M or NF-H drives de novo assembly of neurofilaments in IF⁻ SW13 cells. SW13 cells were co-transfected with pCMV-NFL and either (A and B) pMSV-NFM or (C and D) pMSV-NFH. (A and C) Immunofluorescence localization of NF-L by affinity-purified rabbit anti-NF-L antibodies followed by fluorescein-conjugated secondary IgG. (B) NF-M and (D) NF-H were colocalized with NF-L in the same NF-L transfected cells shown in A and B using double immunofluorescence microscopy by mouse monoclonal anti-NF-M or NF-H antibodies followed by Texas red-conjugated horse anti-mouse IgG secondary. Bar: (A and B) 16 μ m; (C and D) 10 μ m.

Further, the mouse NF-L and NF-M subunits were fully competent for coassembly with human vimentin in IF⁺ SW13 cells (Fig. 3, A–D), results consistent with earlier transfection experiments in other cells (Monteiro and Cleveland, 1990; Chin et al., 1991). Similarly, in cells expressing low levels of NF-H (i.e., those with dimmer fluorescent signals) NF-H too coaligned into a vimentin filament network (as in other cells; Chin and Liem, 1990). However, as might be expected from its *in vitro* behavior, higher levels of the completely wild type NF-H polypeptide yielded nonfilamentous, punctate aggregates and a partially disrupted the endogenous vimentin network both in SW13 IF⁺ [Fig. 3, E and F] and mouse L cells [Fig. 3, G and H].

We conclude that, since no single NF subunit is capable of forming a homopolymeric filament network in the IF⁻ SW13 cell, this must reflect a network assembly defect in each individual subunit.

NF-M or NF-H Complements In Vivo Network Assembly Incompetence of NF-L

Since NF-L *in vivo* is always expressed in conjunction with other IF subunits (see Nixon and Shea, 1992), the fact that NF-L alone is not sufficient to form an *in vivo* filament network suggests that multiple NF subunits may be required to form a neurofilament network *in vivo*. To examine directly the possibility that neurofilaments *in vivo* are obligate heteropolymers, co-transfection was used to express NF-L with NF-M or NF-H in IF⁻ SW13 cells. In co-transfected cells, NF-L always assembled into a distinctive filamentous network consisting of long individual filaments, as well as thick bundles of filaments (Fig. 4). In extreme cases, lateral association of filaments yielded thick cables of filaments containing both NF-L (cell at lower right in Fig. 4 A) and NF-M (Fig. 4 B). In all cases, both NF-M (Fig. 4 B) and NF-H

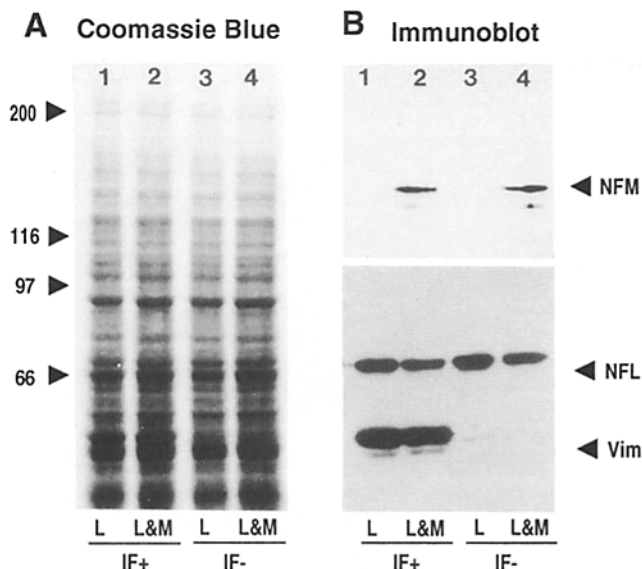


Figure 5. A sub-stoichiometric amount of NF-M is able to drive assembly of NF-L into neurofilaments in vivo. (A) 40 μ g of proteins from cytoskeletal extracts of IF⁺ (lanes 1 and 2) and IF⁻ (lanes 3 and 4) SW13 cells transfected either with pCMV-NFL alone (L; lanes 1 and 3) or co-transfected with pCMV-NFL and pMSV-NFM (L&M; lanes 2 and 4) were separated by SDS-PAGE and stained with Coomassie blue. (B) Duplicate gels were immunoblotted and reacted with mouse mAbs to NF-L and vimentin or NF-M, followed by ¹²⁵I-labeled goat anti-mouse secondary antibody to visualize proteins by autoradiography. (For detection of NF-M, 80- μ g aliquots of proteins were loaded on each lane.) The molecular weight markers (in kD) are indicated on the left and the positions of NF-L, NF-M, and vimentin are indicated on the right.

(Fig. 4 D) colocalized completely in heteropolymers with NF-L (Fig. 4, A and C). Expression of NF-M with NF-H in IF⁻ SW13 cells resulted in coaggregation of both NF subunits into nonfilamentous punctate structures (not shown).

One explanation for how NF-M or NF-H complements network assembly of NF-L would be that coexpression somehow enhanced the level of accumulated NF-L, thereby driving de novo filament assembly. To address this, the relative levels of NF-L accumulated in IF⁺ and IF⁻ cells were determined. Proteins from cells 40 h after transfection with either NF-L alone or NF-L and NF-M were separated by SDS-PAGE (Fig. 5 A) and immunoblotted to visualize vimentin, NF-L and NF-M (Fig. 5 B). Dilutions of known amounts of NF-L, NF-M, NF-H, and vimentin were immunoblotted in parallel to provide accurate quantitative standards (not shown). The number of cells expressing NF-L was as assayed by immunofluorescence microscopy and found to be comparable in all cases (between 15 and 20%). The immunoblots revealed that in both IF⁺ (Fig. 5, lanes 1, 2) and IF⁻ (lanes 3, 4) cells, the level of NF-L accumulation was almost identical. The failure of NF-L alone to self assemble could not be accounted for by lower abundance; in fact, when NF-L was coexpressed with NF-M (Fig. 5 B, lanes 2, 4), it accumulated to a lower level than in cells expressing NF-L alone (Fig. 5 B, lanes 1, 3). Moreover, quantification of NF-L and NF-M in cells expressing both

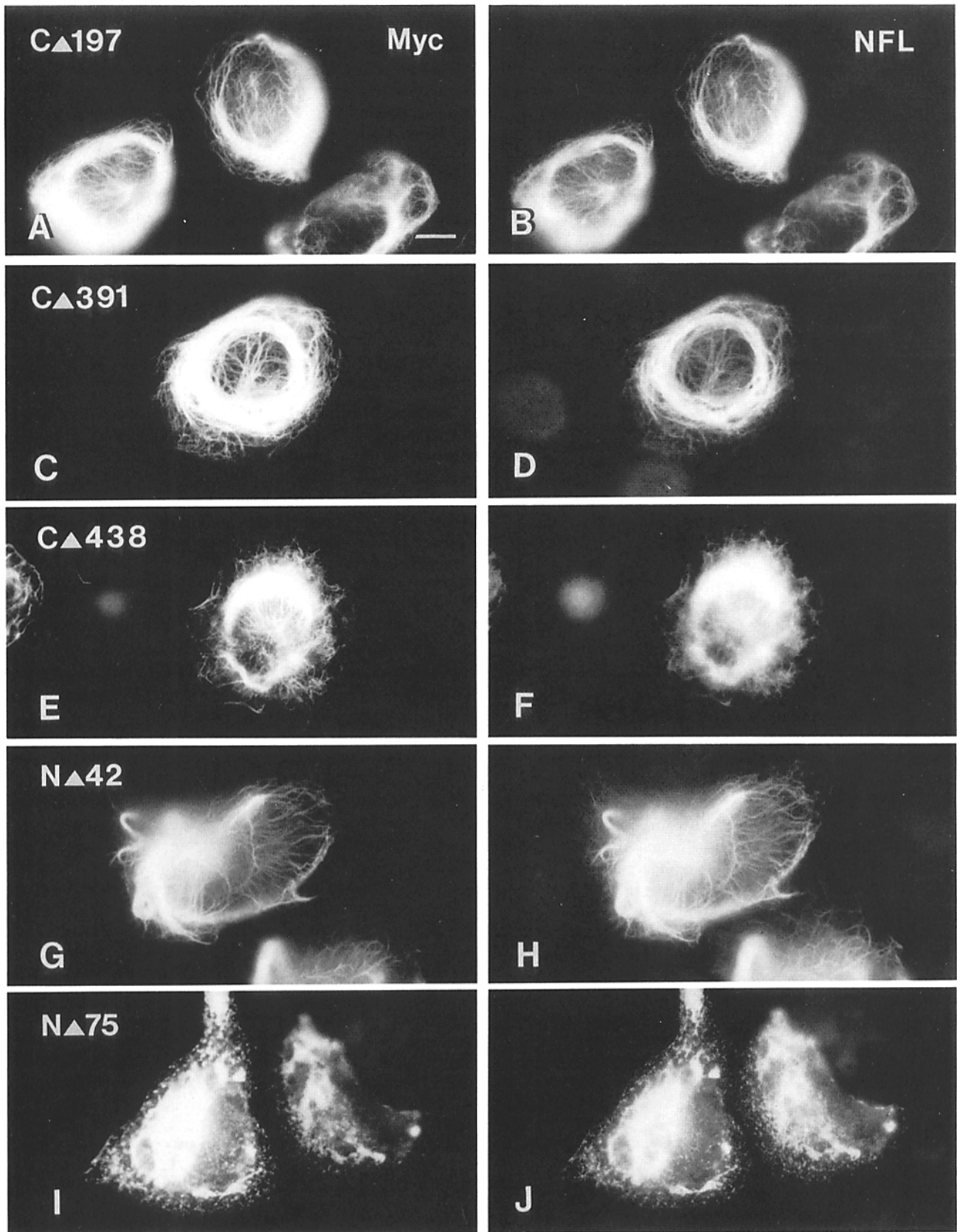
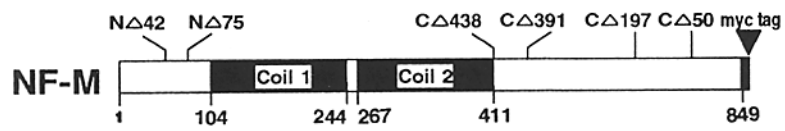
subunits revealed that (in molar amounts) NF-M constituted only ~10% of NF-L. Since calcium phosphate-mediated co-transfection results in a relatively uniform ratio of transgene expression in each transfected cell (as judged by relative fluorescent intensities; see also Kingston et al., 1989), the molar ratios of the subunits in the extracts probably reflect the molar ratios of NF subunits in most of the individual transfected cells. Thus, coexpression of NF-M is sufficient to complement network defective NF-L, even when the total concentration of NF subunits is lower than in cells expressing NF-L alone.

We conclude that (a) individual NF-subunits are incompetent to self-assemble into an extended filament network in vivo; (b) NF-L is necessary but not sufficient to drive neurofilament network assembly in vivo; and (c) incorporation of substoichiometric amounts of NF-M are able to complement the network assembly defect of NF-L alone.

Sequences within the Amino-terminal Head, but Not the Carboxyl-terminal Tail, of NF-M and NF-L Are Essential for De Novo Assembly of Neurofilaments In Vivo

To examine what domain(s) of NF-M is necessary for complementation of de novo assembly of NF-L into a filament network in vivo, a series of carboxyl-terminal or amino-terminal truncation mutants of NF-M were co-transfected into IF⁻ SW13 cells along with the wild-type NF-L. Each mutant NF-M was epitope tagged at its carboxy terminus and the mutant was localized along with NF-L using double immunofluorescence microscopy. When NF-M missing 50 (NFM-C Δ 50, not shown), 197 (NFM-C Δ 197, Fig. 6 A), or 391 (NFM-C Δ 391, Fig. 6 C) amino acids from the carboxyl-terminus was expressed with wild-type NF-L, the mutant NF-M coassembled with NF-L and drove the assembly of a de novo neurofilament network. Even truncation of the entire tail (NFM-C Δ 438, Fig. 6 E) still complemented network assembly from NF-L. Further, among the different NF-M mutants and the wild-type NF-M, there were no consistent differences in the actual morphology of the assembled networks. The images shown (Fig. 6, A-F) give representative examples of various morphologies of networks that are observed with all NF-M subunits. In particular, filament bundling into thick cables is a prominent feature. Further truncation into the rod domain of NF-M (e.g., NFM-C Δ 443, not shown) did not support NF-L assembly. These results demonstrate that the entire tail domain of NF-M is dispensable for driving network assembly of NF-L in an in vivo context.

Next, the necessity of the amino-terminal head region of NF-M for de novo filament assembly with NF-L was examined. When NF-L was expressed with NF-M deleted 42 amino acids from its amino terminus (NFM-N Δ 42), NFM-N Δ 42 was able to coassemble with NF-L and drive the assembly of a filament network in complete absence of vimentin (Fig. 6, G and H). However, when NF-M missing 75 amino-terminal amino acids (NFM-N Δ 75) was expressed with the wild-type NF-L, both NFM-N Δ 75 and NF-L colocalized to nonfilamentous, punctate aggregates (Fig. 6, I and J). Thus, amino acid sequences contained within the region between Ser₄₂ and Ser₇₅ in NF-M are essential for



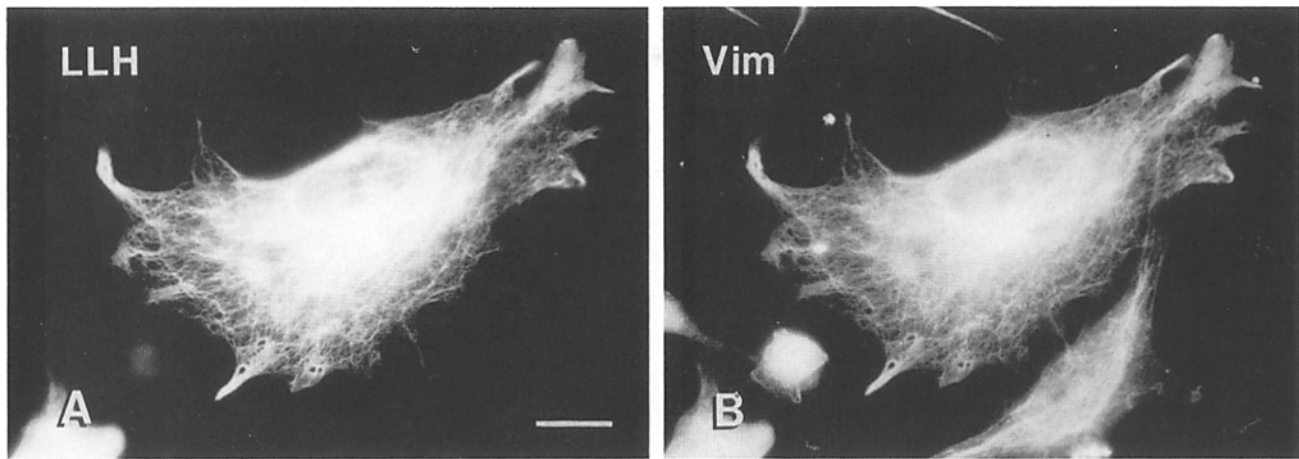


Figure 7. The hybrid NF-subunit LLH efficiently coassembles with vimentin into a filament network. L-cells were transiently transfected with pMSV-LLH. (A) LLH-hybrid was localized with a mouse monoclonal anti-*myc* antibody and (B) vimentin in the same transfected cells was localized by goat anti-vimentin polyclonal antibody. Bar, 10 μ m.

driving assembly of NF-L into a neurofilament network *in vivo*.

The roles of the NF-L amino-terminal head and carboxyl-terminal tail in *de novo* assembly of neurofilaments were also examined. To test if any specific sequence within the entire carboxyl-terminal tail region of NF-L is essential for assembly of a filament network *in vivo*, a hybrid NF-gene was constructed to encode a subunit in which the carboxyl-terminal tail of NF-L was replaced with the entire carboxyl-terminal tail of NF-H (Fig. 1 D). The hybrid protein, named LLH (the letters denote the subunit origin of the head, rod and tail, respectively) coassembles with the endogenous vimentin into cytoplasmic filament networks without any obvious signs of filament disruption or formation of nonfilamentous aggregates (Fig. 7, A and B). Thus, the assembly properties of the LLH hybrid in the presence of vimentin better approximates NF-L than NF-H, since at comparable levels of expression NF-H disrupts filament networks in a significant number of cells. In IF⁻ SW13 cells, the expression of the LLH hybrid alone (not shown) or co-expression with wild-type NF-L (Fig. 8, A and B) results in localization of the LLH hybrid into nonfilamentous aggregates that also contain NF-L. However, coexpression of LLH with wild-type NF-M yields assembly of LLH (Fig. 8 C) and NF-M (Fig. 8 D) into a cytoplasmic filament "network" composed of both long and short fragments of individual filaments, as well as small bundles of filaments. (LLH containing networks reproducibly show less extensive filament assembly than those constructed from NF-L, a feature most likely due, at least in part, to lower expression of MSV-promoted LLH. When MSV-NFL

is used to express NF-L along with NF-M, a very similar filament array consisting of short and long filament fragments and small bundles is produced [not shown].)

From these results, we conclude that (a) no specific sequence within the entire tail region of NF-L is required for *de novo* filament assembly *in vivo*; (b) sequences within the NF-H tail are not directly involved in the assembly process; and (c) the NF-M and NF-H tail regions do not prevent an otherwise assembly competent NF subunit from filament assembly.

To examine the influence of sequences contained in the amino-terminal head region of NF-L, NF-L truncation mutants in which either 22 (NFL-N Δ 22) or 89 (NFL-N Δ 89) amino acids were removed from the 93-amino acid amino-terminal head domain were individually coexpressed with the wild-type NF-M (each NF-L subunit was also epitope tagged at its carboxy terminus). In SW13 IF⁺ cells, where either truncated NF-L subunit accumulated to a low level, expression of NFL-N Δ 22 or NFL-N Δ 89 resulted in complete coassembly with endogenous vimentin into the filament network (not shown). In SW13 IF⁻ cells, expression of NFL-N Δ 22 with NF-M also resulted in assembly of a filament network consisting completely of copolymers of NFL-N Δ 22 and NF-M (Fig. 9, A and B). However, the parallel experiment with NFL-N Δ 89 invariably yielded nonfilamentous punctate aggregates containing both NFL-N Δ 89 and NF-M (Fig. 9, C and D). Thus, as with NF-M, sequences within the amino-terminal head region (between Pro₂₂ and Pro₈₉) of NF-L are necessary for *de novo* assembly of filaments in an *in vivo* context.

Figure 6. Sequences within the amino-terminal head but not carboxyl-terminal tail of NF-M are essential for complementing *de novo* assembly of NF-L *in vivo*. IF⁻ SW13 cells were co-transfected with pCMV-NFL and a series of either (A-F) carboxyl-terminal or (G-J) amino-terminal truncation mutants of NF-M. Numbers indicate deletion endpoints from either amino or carboxyl termini and the approximate borders of deletions are shown on the schematic of NF-M protein at the top. All of the NF-M mutant proteins contain a 12-amino acid *myc*-tag at the extreme carboxyl-terminus. (A, C, E, G, and I) Mutant NF-M subunits were localized by immunofluorescence microscopy using mouse monoclonal anti-*myc*-tag antibodies followed by fluorescein-conjugated horse anti-mouse IgG secondary. (B, D, F, H, and J) NF-L subunits in same transfected cells as in A, C, E, G, and I were localized by double immunofluorescence microscopy using affinity-purified rabbit anti-NF-L antibody followed by Texas red-conjugated goat anti-rabbit IgG secondary. Bar, 10 μ m.

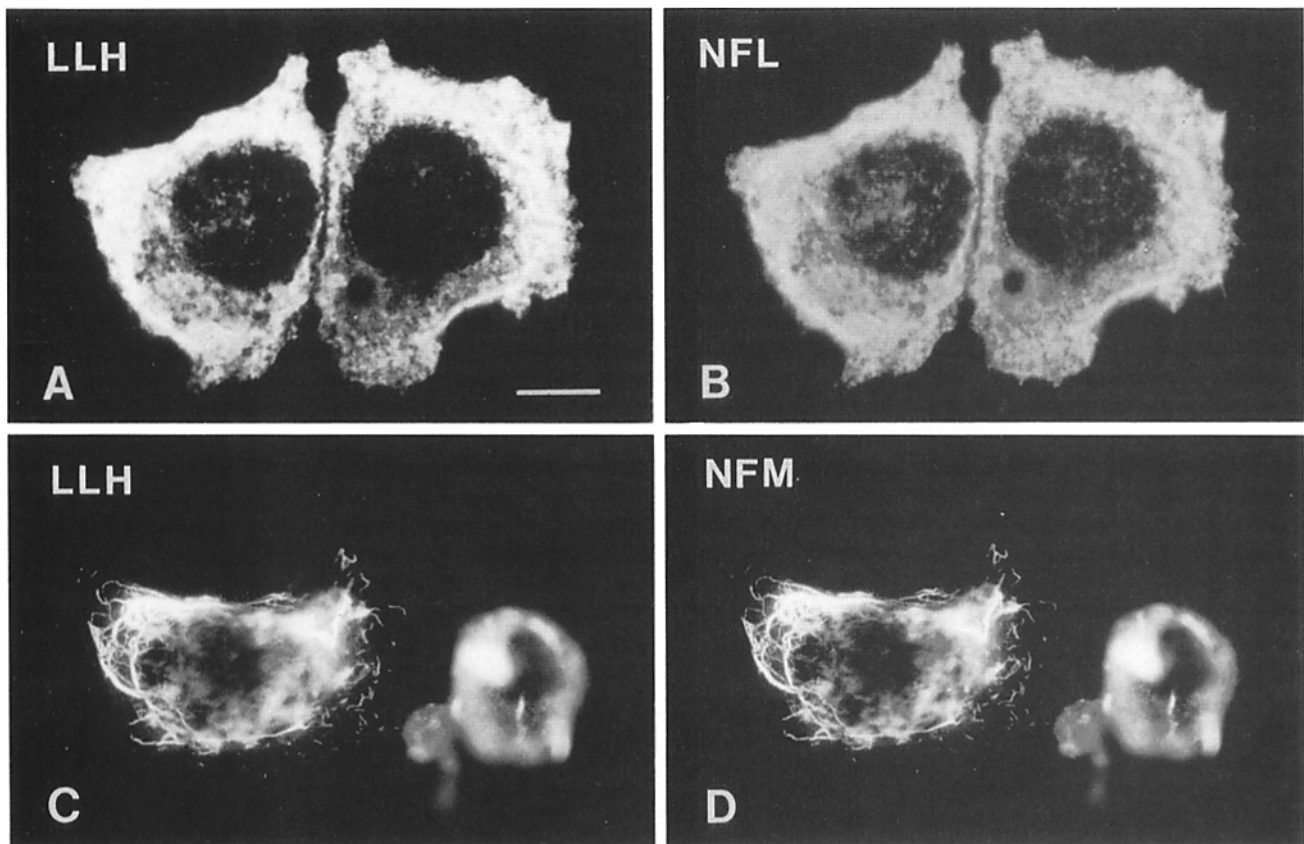


Figure 8. LLH complements de novo assembly incompetence of NF-M. IF⁻ SW13⁻ cells were co-transfected with pMSV-LLH and either (A and B) pMSV-NFL or (C and D) pMSV-NF-M. (A and C) The LLH-hybrid was localized by affinity-purified rabbit anti-*myc* polyclonal antibodies followed by fluorescein-conjugated secondary. Double immunofluorescence microscopy was used to localize (B) NF-L and (D) NF-M by mouse mAbs to either NF-L or NF-M, followed by Texas red-conjugated secondary. Bar, 10 μm.

Assembly of Neurofilament Networks in IF⁻ Oligodendrocytes of Transgenic Mice Expressing Both NF-L and NF-M, but Not Either Subunit Alone

To examine the de novo assembly properties of NF-L and NF-M in another *in vivo* context, we exploited two sets of transgenic mice that express MSV-promoted transgenes encoding either wild-type mouse NF-L (Monteiro et al., 1991; Xu et al., 1993) or a mouse NF-M subunit in which the last 50 carboxy-terminal tail amino acids were replaced with a 12-amino acid epitope tag (Wong, P. C., J. Marszalek, and D. W. Cleveland, manuscript in preparation). Unlike the endogenous NF-L and NF-M genes, immunocytochemistry of frozen sections of tissues from transgenic animals revealed that both transgenes are expressed in some non-neuronal cells, including in oligodendrocytes of the lumbar spinal cord (data not shown). Since these cells, which myelinate central nervous system neurons, do not normally express cytoplasmic intermediate filaments (e.g., Peters et al., 1991), we used EM of tissue sections of NF-L or NF-M transgenic animals to examine de novo filament network assembly. Just as in transfected SW13⁻ cells, extended filament arrays were not observable in oligodendrocytes of heterozygotes of either transgenic line (Fig. 10, A and B). However, examination of oligodendrocytes produced by mating these two lines revealed that expression of both NF-L and NF-M yielded prominent, closely spaced bundles of 8–10-nm filaments (Fig. 10 C) that, at this level of resolution, were essentially

indistinguishable from those found in neurons. Since such filament arrays were not found in oligodendrocytes from animals homozygous for either transgene, filament assembly in the NF-L/NF-M doubly transgenic animals cannot be due simply to increased expression of NF polypeptides; rather, it must result from assembly promoting interactions between NF-L and NF-M subunits.

Discussion

By transfection into a IF⁻ cultured cell line and by forcing expression of NF subunits in IF⁻ oligodendrocytes in transgenic mice, we have uncovered the unexpected finding that, despite self assembly *in vitro*, NF-L is not sufficient for assembly of an NF network. Defective by itself, network assembly is restored to NF-L by substoichiometric amounts of NF-M or NF-H, neither of which alone or in combination with each other assemble networks. The obvious conclusion is that *in vivo* neurofilaments are obligate heteropolymers assembled from NF-L and NF-M/NF-H. Since tailless NF-M also complements NF-L assembly, but truncations into the amino-terminal head cannot, sequences in the NF-M head (and rod) provide the essential interaction for network formation by NF-L. Although the requirement of the NF-H head has not been directly tested, its tail (carried by hybrid subunit LLH) does not complement NF-L assembly. Thus, it seems highly likely that for NF-H, too, sequences in the head (and rod) mediate *in vivo* NF-L assembly.

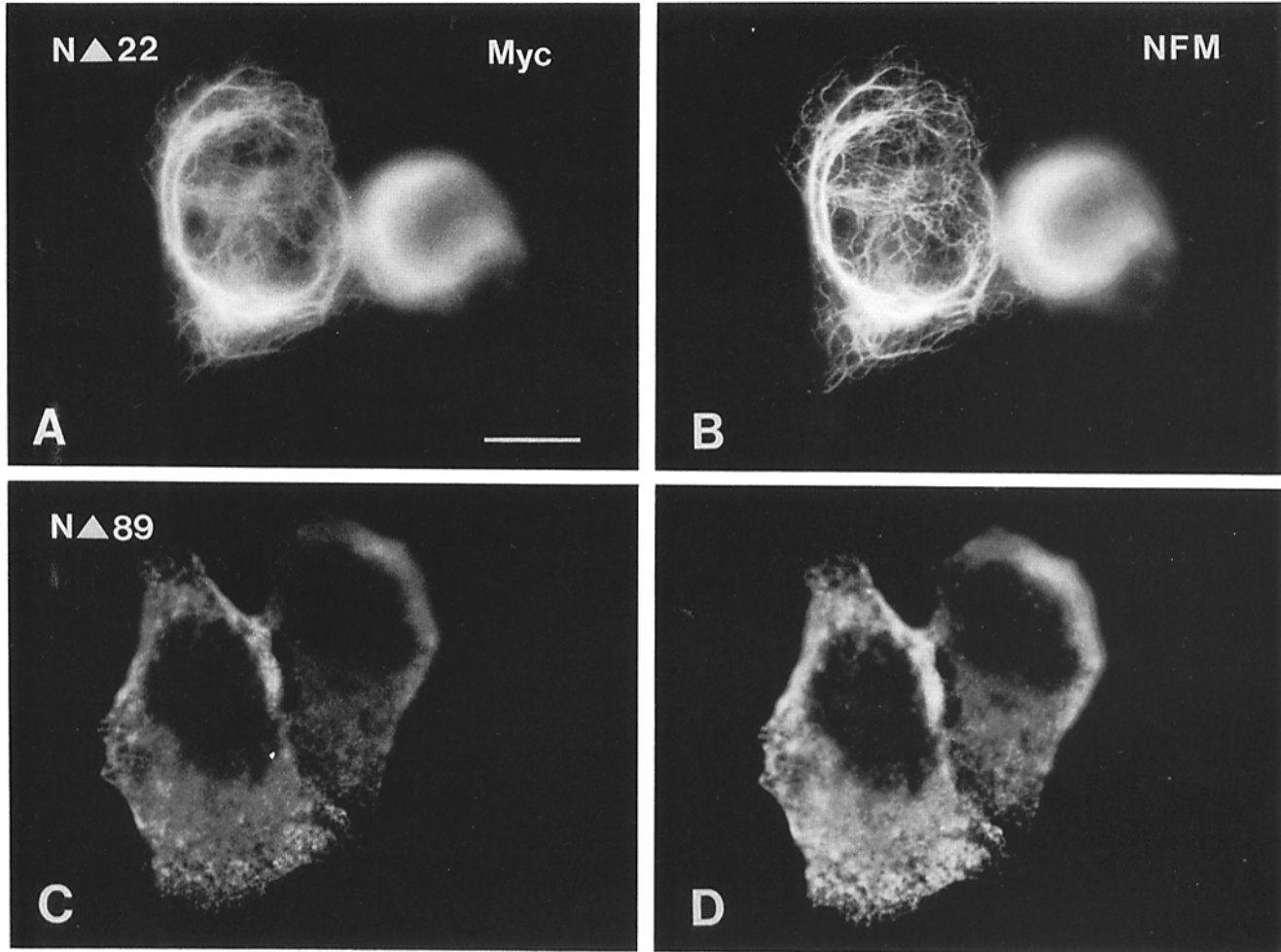
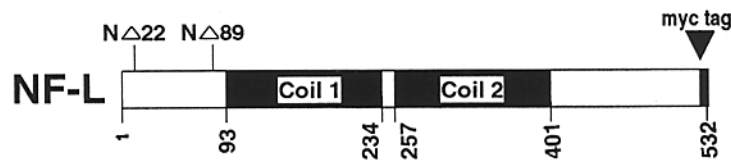


Figure 9. The amino-terminal head of NF-L is essential for de novo coassembly with NF-M. IF⁻ SW13 cells were co-transfected with pMSV-NFM and either (A and B) pMSV-NFL-N Δ 22 or (C and D) pMSV-NFL-N Δ 89. Immunofluorescence microscopy was used to localize (A) NFL-N Δ 22 or (C) NFL-N Δ 89 by affinity-purified rabbit polyclonal antibody followed by fluorescein-conjugated secondary. (B and D) Double immunofluorescence microscopy was used to localize NF-M by mouse monoclonal anti-NF-M antibodies, followed by a Texas red-conjugated secondary. Bar, 10 μ m.

The molecular basis for inability of NF-M and NF-H to form heteropolymeric filaments together *in vivo* is not clear. However, it is reasonable to assume that the carboxyl-terminal tail domains of these subunits do not inhibit the actual assembly process, as shown by LLH/NFM heteropolymeric filament assembly (Fig. 8). Possibly the rod domains of NF-M and NF-H do not interact in a productive manner and may need to interact with NF-L. The structural basis for this possibility is suggested by the fact that unlike the assembly competent type I, II, III, and other IV subunits, neither NF-M nor NF-H rods has the characteristic disruption in the α -helix in coil I (Lees et al., 1988; Myers et al., 1987). However, nothing in the present evidence excludes the alternative that some domain in the NF-L amino-terminal head may also be required for proper coassembly.

That neurofilaments *in vivo* are obligate heteropolymers requiring NF-L and either NF-M or NF-H clearly establishes the functional importance of NF-M and NF-H in the assembly and organization of neurofilaments. An obvious extension of this finding is that the assembly of neurofilaments in neurons may be affected by posttranslational regulation of any one of the NF subunits. Both the obligate heteropolymeric nature and the tendency for these filaments to bundle laterally *in vivo* resemble the properties of keratin filaments (Bader et al., 1991; Lu and Lane, 1990). However, the assembly of neurofilaments and keratins differ in one important area: the keratin filaments are stoichiometric heterodimers requiring one Type I and Type II keratin. In contrast, neurofilaments can apparently accommodate a wide range of NF-subunit ratios, a point of some significance since the NF-

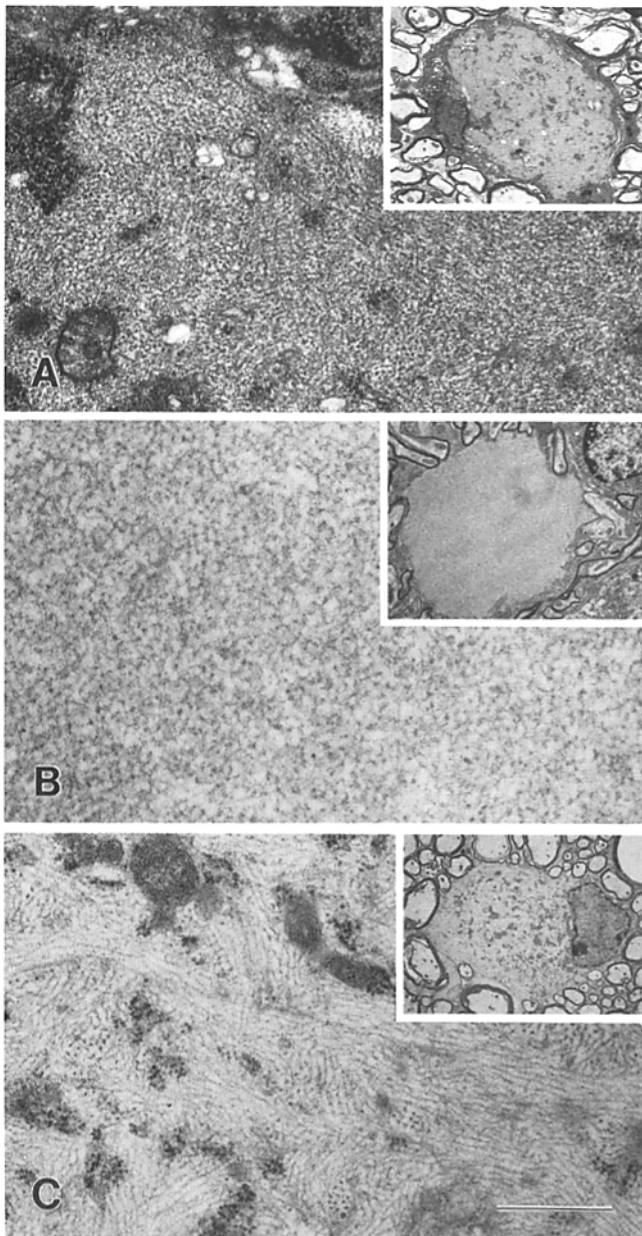


Figure 10. De novo assembly of an NF network in oligodendrocytes of transgenic mice requires expression of both NF-L and NF-M transgenes. Thin section electron micrographs of oligodendrocytes expressing (A) NF-L alone, (B) NF-Mc Δ 50 alone, and (C) both NF-L and NF-Mc Δ 50. The insets show the cells from which A, B, and C were taken. Bar: 750 nm; (insets) 6 μ m.

subunit ratios do change dramatically during neuronal development (see Nixon and Shea, 1992). Whether the requirement for heteropolymerization acts at the dimer, tetramer or higher order oligomer level has not yet been determined, although crosslinking experiments (Carden and Eagles, 1986) and antibody labeling of filaments assembled in vitro (Mulligan et al., 1991) are most easily explained by NF-L/NF-M or NF-L/NF-H heterodimers.

Another feature that was not revealed by in vitro reassembly studies or previous transfection studies is the propensity for neurofilaments assembled in vivo to interact laterally to form bundles. Although some lateral interaction of filaments is seen with vimentin alone (Fig. 2 D) or when NF-subunits

are coexpressed with vimentin (Fig. 3, A and C), the bundles are neither as striking or abundant as that observed for NF assembled in the absence of vimentin (e.g., compare Fig. 3, A and C with Fig. 4, A, C, and E). Possibly this is because vimentin (and other Type III subunits) contain a region within the carboxyl-terminal domain that prevents filaments from laterally associating (Kouklis et al., 1991). On the other hand, neurofilaments may possess an inherent tendency to interact laterally, thereby allowing the carboxyl-terminal tail regions of NF-M and NF-H to modulate lateral spacing between filaments by steric and electrostatic interactions. This hypothesis is consistent with the observation that the carboxyl-terminal tail region of both NF-M and NF-H extend from the surface of the filaments (Hisanaga and Hirokawa, 1988). However, if this is true, what remains unclear is why neurofilaments have little lateral affinity in vitro.

While the carboxyl-terminal tails are likely to be involved in regulation of higher order interaction between filaments and other cellular structures, the tails are not likely to be involved directly in actual assembly of neurofilaments in vivo. Even a completely tailless NF-M stimulates network coassembly with NF-L. At first glance, the efficient assembly of tailless NF-M (NFM-C Δ 438) with NF-L into a filament network may seem at odds with a previous report that this truncated NF-M is a dominant assembly disrupter (Wong and Cleveland, 1990). However, this is probably related to the differences in the level of expression after transfection into different cells; higher levels disrupt neurofilament arrays. Although NFL-N Δ 89 has also been reported to be an assembly defective mutant (Gill et al., 1990), differences in the levels of mutant accumulation also probably account for coassembly of this "headless" NF-L mutant with the wild-type vimentin array observed here and by others (Chin and Liem, 1991). Overall, the current results are consistent with effects of carboxyl-terminal tail mutations on other filament types which collectively show that the amino-terminal head region is much more directly involved in filament assembly than are the carboxyl-terminal tail domains (Raats et al., 1992, 1991, 1990; Bader et al., 1991; Lu and Lane, 1990; Kaufmann et al., 1985; also see Stewart, 1993). One question that remains untested is whether a filament network can be assembled from tailless NF-L and NF-M. For other IF subunits, several studies collectively suggest that filament assembly with tailless mutants may require presence of wild-type subunits (Albers and Fuchs, 1987; Lu and Lane, 1990; Raats et al., 1991; Eckelt et al., 1992; also see Coulombe, 1993).

The importance of the amino-terminal head region in assembly of neurofilaments in vivo has particular relevance to the regulation of neurofilament assembly in vivo. Direct involvement of amino-terminal head region of IF-subunits in filament assembly is indicated by deletion mutagenesis experiments on many IF subunits (Raats et al., 1992, 1991, 1990; Bader et al., 1991; Lu and Lane, 1990; Kaufmann et al., 1985; also see Stewart, 1993), including neurofilaments (Gill et al., 1990; Wong et al., 1990; Chin et al., 1991). The importance of the head domains for neurofilament assembly is further underscored by our finding that truncation of 75% of the NF-M head (NFM-N Δ 75) fails to complement NF-L assembly, although it can coassemble with self-assembly competent vimentin (Wong and Cleveland, 1990). Further, the amino-terminal head domain of Type III, IV, and V

subunits are known to be substrates for phosphorylation in vivo and phosphorylation of this region either inhibits assembly or causes disassembly of filaments both in vitro and in vivo (Ando et al., 1989; Evans, 1989; Inagaki et al., 1989; Kitamura et al., 1989; Chou et al., 1990; Hisanaga et al., 1990; Peter et al., 1990; Sihag and Nixon, 1990, 1991). For the NF subunits, major in vivo phosphorylation sites on the amino-terminal head domain are either located within (Ser₅₅ on NF-L and a phosphopeptide starting at Ser₄₄ on NF-M; Sihag and Nixon, 1990, 1991) or are very near (a phosphopeptide starting at Ser₂₅ on NF-M; Sihag and Nixon, 1990) the sequences essential for in vivo neurofilament assembly. Additionally, the phosphate on Ser₅₅ of NF-L displays rapid turnover immediately after NF-L synthesis in neurons (Sihag and Nixon, 1991). Moreover, both NF-L and NF-M isolated from rat spinal cord are posttranslationally modified by addition of O-linked N-acetylglucosamine moieties (Dong et al., 1993). Three of four sites identified lie in the amino-terminal head region and all of three of these are located within (Ser₂₇ on NF-L and Thr₄₈ on NF-M) or near (Thr₂₁ on NF-L) the domain essential for in vivo neurofilament assembly. Collectively, it seems very likely that modification of the head domains on NF subunits affects assembly. Among the interesting possibilities for regulation include blocking premature assembly prior to transport into neurites. It remains for future efforts to define more clearly how each modification ultimately affects network assembly.

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References

- Albers, K., and E. Fuchs. 1987. The expression of mutant epidermal keratin cDNAs in simple epithelial and squamous cell carcinoma lines. *J. Cell Biol.* 105:791-806.
- Ando, S., K. Tanabe, Y. Gonda, C. Sato, and M. Inagaki. 1989. Domain- and sequence-specific phosphorylation of vimentin induces disassembly of the filament structure. *Biochemistry.* 28:2974-2979.
- Bader, B. L., T. M. Magin, M. Freudenmann, S. Stumpp, and W. W. Franke. 1991. Intermediate filaments formed de novo from tail-less cytokeratins in the cytoplasm and in the nucleus. *J. Cell Biol.* 115:1293-1307.
- Balin, B. J., and V. M.-Y. Lee. 1991. Individual neurofilament subunits reassembled in vitro exhibit unique biochemical, morphological and immunological properties. *Brain Res.* 556:196-208.
- Carden, M. J., and Eagles, P. A. M. 1986. Chemical crosslinking analyzes of ox neurofilaments. *Biochem. J.* 234:587-591.
- Chin, S. S., and R. K. H. Liem. 1990. Transfected NF-H coassembles with vimentin in nonphosphorylated form. *J. Neurosci.* 10:3714-3726.
- Chin, S. S., P. Macioce, and R. K. H. Liem. 1991. Effects of truncated neurofilament proteins on the endogenous intermediate filaments in transfected fibroblasts. *J. Cell Sci.* 99:335-350.
- Chou, C.-H., E. Rosevear, and R. D. Goldman. 1990. Phosphorylation and disassembly of intermediate filaments in mitotic cells. *Proc. Natl. Acad. Sci. USA.* 86:1885-1889.
- Cleveland, D. W., M. J. Monteiro, P. C. Wong, S. R. Gill, J. D. Gearhart, and P. N. Hoffman. 1991. Involvement of neurofilaments in the radial growth of axons. *J. Cell Sci.* 15: 85-95.
- Côté, F., J.-F. Collard, and J.-P. Julien. 1993. Progressive neuropathy in transgenic mice expressing the human neurofilament heavy gene: A mouse model of amyotrophic lateral sclerosis. *Cell.* 73:35-46.
- Dong, D. L.-Y., Z.-S. Xu, M. R. Chevrier, R. J. Cotter, D. W. Cleveland, and G. W. Hart. 1993. Glycosylation of mammalian neurofilaments: Localization of multiple O-linked N-acetylglucosamine moieties on neurofilament polypeptides L and M. *J. Biol. Chem.* 268:16679-16687.
- Eckelt, A., H. Herrmann, and W. Franke. 1992. Assembly of tail-less mutant of the intermediate protein, vimentin, in vitro and in vivo. *Eur. J. Cell Biol.* 58:319-330.
- Evans, R. M. 1989. Phosphorylation of vimentin in mitotically selected cells. In vitro cAMP-independent kinase and calcium stimulated phosphatase activity. *J. Cell Biol.* 108:67-78.
- Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell Biol.* 5:3610-3616.
- Fliegener, K. H., G. Y. Ching, and R. K. H. Liem. 1990. The predicted amino acid sequence of α -internexin is that of a novel neuronal intermediate filament protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:749-755.
- Gardner, E. E., D. Dahl, and A. Bignami. 1984. Formation of 10 nm filaments from 150 kDa neurofilament protein in vitro. *J. Neurosci. Res.* 11:145-155.
- Giesler, N., and K. Weber. 1981. Self-assembly in vitro of 68,000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate size filaments. *J. Mol. Biol.* 151:565-571.
- Gill, S. R., P. C. Wong, M. J. Monteiro, and D. W. Cleveland. 1990. Assembly properties of dominant and recessive mutations in the small mouse neurofilament (NF-L) subunit. *J. Cell Biol.* 111:2005-2019.
- Graham, R., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456-467.
- Hirokawa, N., M. A. Glicksman, and M. B. Willard. 1984. Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton. *J. Cell Biol.* 98:1523-1536.
- Hisanaga, S.-I., Y. Gonda, M. Inagaki, A. Ikai, and N. Hirokawa. 1990. Effects of phosphorylation of neurofilament L protein on filamentous structure. *Cell Regul.* 1:237-248.
- Hisanaga, S.-I., and N. Hirokawa. 1988. Structure of the peripheral domains of neurofilaments revealed by low angle rotary shadowing. *J. Mol. Biol.* 20:297-305.
- Inagaki, M., Y. Nishi, K. Nishizawa, M. Matsuyama, and C. Sato. 1989. Site-specific phosphorylation induces disassembly of vimentin filaments in vitro. *Nature (Lond.)* 328:649-652.
- Julien, J.-P., F. Côté, L. Beaudet, M. Sidky, D. Flavell, F. Grosveld, and W. Mushynski. 1988. Sequence and structure of the mouse gene encoding for the largest neurofilament subunit. *Gene (Amst.)* 68:307-314.
- Kaufmann, E., K. Weber, and N. Geisler. 1985. Intermediate filament forming ability of desmin derivatives lacking either the amino-terminal 67 or the carboxyl-terminal 27 residues. *J. Mol. Biol.* 185:733-742.
- Kingston, R. E. 1990. Transfection of DNA into eukaryotic cells. In *Current Protocols in Molecular Biology*. F. Ausubel, R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl, editors. John Wiley and Sons Inc., New York.
- Kitamura, S., S. Ando, M. Shibata, K. Tanabe, C. Sato, and M. Inagaki. 1989. Protein kinase C phosphorylation of desmin at four serine residues within the non- α -helical head domain. *J. Biol. Chem.* 264:5674-5678.
- Kouklis, P. D., T. Papamarcaki, A. Merdes, and S. D. Georgatos. 1991. A potential role for the COOH-terminal domain in the lateral packing of type III intermediate filaments. *J. Cell Biol.* 114:773-786.
- Laemmli, U. K. 1970. Cleavage structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lees, J. F., P. S. Shneidman, S. F. Skutz, M. J. Carden, and R. A. Lazzarini. 1988. The structure and organization of heavy neurofilament subunit (NF-H) and gene encoding it. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1947-1955.
- Liem, R. K. H., and S. B. Hutchinson. 1982. Purification of individual components of the neurofilament triplet: filament assembly from the 70,000 dalton subunit. *Biochemistry.* 21:3221-3226.
- Lopata, M. A., and D. W. Cleveland. 1987. In vivo microtubules are copolymers of available β -tubulin isotypes: localization of each of the six vertebrate β -tubulin isotypes using polyclonal antibodies elicited by synthetic peptide antigens. *J. Cell Biol.* 105:1707-1720.
- Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb. 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with dimethylsulfoxide or glycerol shock treatment. *Nucleic Acids Res.* 12:5707-5717.
- Lu, X., and E. B. Lane. 1990. Retrovirus-mediated transgenic keratin expression in cultured fibroblasts: Specific domain functions in keratin stabilization and filament formation. *Cell.* 62:681-696.
- Monteiro, M. J., and D. W. Cleveland. 1990. Expression of NF-L and NF-M in fibroblasts reveals coassembly of neurofilament and vimentin subunits. *J. Cell Biol.* 108:579-593.
- Mulligan, L., Balin, B. J., Lee, V. M.-Y., and Ip, W. 1991. Antibody labeling of bovine neurofilaments: implication on the structure of neurofilament sidearms. *J. Struct. Biol.* 106:145-160.
- Myers, M. W., R. A. Lazzarini, V. M. Y. Lee, W. W. Schlaepfer, and D. L. Nelson. 1987. The human mid size neurofilament subunit: a repeated protein sequence and the relation ship of its gene to the intermediate filament gene family. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1617-1626.
- Nixon, R. A., and T. B. Shea. 1992. Dynamics of neuronal intermediate filaments: a developmental perspective. *Cell Motil. Cytoskeleton.* 22:81-91.
- Peter, M., J. Nakagawa, M. Doree, J. C. Ley, and R. D. Goldman. 1990. In vitro disassembly of nuclear lamina and M-phase-specific phosphorylation of lamins by *cdc2* kinase. *Cell.* 61:591-602.

- Peters, A., S. Palay, and H. de F. Webster. 1991. The fine structure of the nervous system. 3rd Edition. Oxford University Press, New York. 296-297.
- Ratts, J. M. H., F. R. Pieper, W. T. Vree Egberts, K. N. Verrijp, F. C. Ramaekers, and H. Bloemendal. 1990. Assembly of amino-terminally deleted desmin in vimentin-free cells. *J. Cell Biol.* 90:1971-1985.
- Ratts, J. M. H., J. B. Henderik, M. Verdijk, F. L. G. van Oort, W. L. Gerards, F. C. Ramaekers, and H. Bloemendal. 1991. Assembly of carboxy-terminally deleted desmin in vimentin-free cells. *Eur. J. Cell Biol.* 56:84-103.
- Ratts, J. M. H., W. L. H. Gerards, M. I. Schruder, C. Grund, J. B. J. Henderik, I. L. Henderiks, F. C. Ramaekers, and H. Bloemendal. 1992. Biochemical and structural aspects of transiently and stably expressed mutant desmin in vimentin-free and vimentin containing cells. *Eur. J. Cell Biol.* 58:108-127.
- Sarria, A., S. Nordeen, and R. Evans. 1990. Regulated expression of vimentin cDNA in the presence and absence of preexisting vimentin filament network. *J. Cell Biol.* 111:553-565.
- Shneidman, P. S., M. J. Carden, J. F. Lees, and R. A. Lazzarini. 1988. The largest murine neurofilament protein (NF-H): Repeated phosphorylation sites. *Mol. Brain Res.* 4:217-231.
- Sihag, R. K., and R. A. Nixon. 1990. Phosphorylation of the amino terminal head domain of the middle molecular mass 145 kDa subunit of neurofilaments: evidence for regulation by second messenger dependent protein kinases. *J. Biol. Chem.* 265:4166-4171.
- Sihag, R. K., and R. A. Nixon. 1991. Identification of Ser-55 as a major protein kinase A phosphorylation site on the 70 kDa subunit of neurofilaments: early turnover during axonal transport. *J. Biol. Chem.* 266:18861-18867.
- Smith, P. K., P. I. Krhon, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzaus, E. K. Fujimoto, N. M. Goetze, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76-85.
- Sommers, C. L., S. E. Heckford, J. M. Skerker, P. Worland, J. A. Torri, E. W. Thompson, S. W. Byers, and E. P. Gelmann. 1992. Loss of epithelial markers and acquisition of vimentin expression in adriamycin- and vinblastin-resistant human breast cancer cell lines. *Cancer Res.* 52:5190-5197.
- Steinert, P. M., and D. R. Roop. 1988. Molecular and cell biology of intermediate filaments. *Annu. Rev. Biochem.* 57:593-625.
- Stewart, M. 1993. Intermediate filament structure and assembly. *Cur. Opin. Cell Biol.* 5:3-11.
- Willard, M., and C. Simon. 1981. Antibody decoration of neurofilaments. *J. Cell Biol.* 89:198-205.
- Wong, P. C., and D. W. Cleveland. 1990. Characterization of dominant and recessive assembly-defective mutations in mouse neurofilament NF-M. *J. Cell Biol.* 111:1987-2003.
- Xu, Z.-S., L. Cork, J. W. Griffin, and D. W. Cleveland. 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., C. Itakura, and M. Mizutani. 1991. Hereditary hypertrophic axonopathy with neurofilament deficiency in a mutant strain of the Japanese quail. *Acta Neuropathol.* 82:427-434.
- Zackroff, R. V., W. W. Idler, P. M. Steinert, and R. D. Goldman. In vitro reconstitution of intermediate filaments from mammalian neurofilament triplet peptides. *Proc. Natl. Acad. Sci. USA.* 79:754-757.