Expression of NF-L and NF-M in Fibroblasts Reveals Coassembly of Neurofilament and Vimentin Subunits

Mervyn J. Monteiro and Don W. Cleveland
The Johns Hopkins University, School of Medicine, Department of Biological Chemistry, Baltimore, Maryland 21205

Abstract. We have used transient and stable DNA transfection to force synthesis of the mouse NF-L and NF-M genes in nonneuronal cultured animal cells. When the authentic NF-L gene (containing 1.7 kb of sequences 5' to the transcription initiation site) was transfected into L cells, correctly initiated NF-L mRNA was produced from the transfected gene but not the endogenous NF-L genes. Therefore, the normal restriction of NF-L expression to neurons cannot derive exclusively from absence in nonneuronal cells of neuron-specific transcription factors. When the NF-L coding region was linked to the strong promoter from Moloney Murine Sarcoma virus, we obtained high levels of synthesis of NF-L subunits (accumulating to as much as 9% of cell protein in stable cell lines). Although NF-L and NF-M polypeptides are normally expressed exclusively in postmitotic neurons, NF-L or NF-M polypeptides expressed in fibroblasts were efficiently assembled into intermediate filament arrays, thus demonstrating the competence of both NF-L and NF-M to assemble in vivo in the absence of additional neuron-specific factors. As judged by immunofluorescence localization and by the alteration in the solubility of the endogenous vimentin filaments, filaments containing NF-L appeared to be copolymers with vimentin. Neither the alteration in the properties of the vimentin array nor the accumulation of NF-L to a level that made it the second most abundant cellular protein (after actin) had any observable effect on cell viability or growth rate.

The cytoskeleton of eukaryotic cells is a dynamic structure composed of microtubules, microfilaments, and intermediate filaments (IFs). The function of microtubules and microfilaments in cellular processes is well understood. However, for IFs, in spite of an increasingly large collection of primary sequence data for most of the members of the IFs, the function of IFs remain poorly characterized. DNA sequence information, together with biochemical and immunological data, has established that IFs are comprised of a heterogeneous group of proteins that have been subdivided into six classes: vimentin (expressed in mesenchymal cells), keratin (expressed in epithelial cells), desmin (expressed in muscle cells), glial fibrillary acidic protein (GFAP) (expressed in glial cells), lamins (expressed in all cell types), and neurofilaments (NFs) (expressed in neuronal cells). In addition to these accepted classes, an additional vimentin-like IF protein expressed in a subset of neurons has recently been described (Portier et al., 1984; Parysek and Goldman, 1987; Leonard et al., 1988).

All IFs contain a highly conserved central α-helical coiled-coiled domain of ~310 residues, flanked by two nonhelical segments of variable length and sequence at the amino- and carboxy-terminal regions, the so called 'head' and 'tail' domains (for reviews see Franke, 1987; Geisler and Weber, 1986). The amino- and carboxy-terminal regions play a role both in the end-to-end and in lateral interactions of filament polymerization (Geisler and Weber, 1982; Steinert et al., 1983; Traub and Vorgias, 1983; Kaufmann et al., 1985). However, an in vitro assembly experiment has shown that two derivatives of desmin, one specifically lacking 67 amino-terminal residues and another lacking 27 carboxy-terminal residues, resulted in the polymerization of the latter but not the former into filaments (Kaufmann et al., 1985). Recently, Albers and Fuchs (1987) and van den Heuvel et al. (1987) transfected a series of truncated genes to demonstrate clearly that all of the carboxy-terminal domain may also be deleted from keratins and desmin without disrupting assembly in vivo.

Mammalian NFs are composed of three protein subunits: NF-L, NF-M, and NF-H with apparent molecular masses on SDS-PAGE gels of 68, 150, and 200 kD, respectively. The three NF proteins differ in size primarily due to increasingly long carboxy-terminal extensions. The carboxy-terminal regions of NF-M and NF-H are highly phosphorylated, which results in an anomalous migration of the two proteins on SDS-PAGE gels (Julien and Mushynski, 1982, 1983; Carden et al., 1985). Phosphorylation, especially of NF-H, may play a role in the interaction of NFs with other neuronal components (e.g., microtubules) (Julien and Mushynsiki, 1983; Carden et al., 1985).
Sternberger and Sternberger, 1983; Minami and Sakai, 1985).

The organization of the three NF proteins in the native NF polymer has yet to be determined. However, antibody decoration of nervous tissue suggests that NF-L is a component of the NF backbone and that NF-H is involved in crossbridge formation between NFs (Hirokawa et al., 1984). The disposition of NF-M is less certain, although it is thought to be more closely associated with the NF core than is NF-H (Sharp et al., 1982; Hirokawa et al., 1984). Evidence in support of this interpretation has been obtained from in vitro reconstitution experiments using each of the three NF subunits. Thus, NF-L can self-assemble in vitro to form 10-nm filaments. Thus, NF-L can self-assemble in vitro to form 10-nm filaments (Geisler and Weber, 1981). Although earlier evidence to the contrary for NF-M had been obtained (Geisler and Weber, 1981), it now appears that NF-M can also self-assemble (at least under certain conditions) to form 10-nm filaments (Gardner et al., 1984; Tokutake et al., 1984). Self-assembly of NF-H results in the formation of short curly filaments, suggesting that incorporation of NF-H into the NF backbone may block filament elongation (Geisler and Weber, 1981). Therefore, NF-H may be exclusively involved in crossbridge formation.

The isolation and sequence of recombinant DNA clones for the NF-L, NF-M, and NF-H genes of mouse (Lewis and Cowan, 1985, 1986; Julien et al., 1986; Levy et al., 1987), rat (Julien et al., 1985; Napolitano et al., 1987), and human (Julien et al., 1987; Myers et al., 1987; Lecs et al., 1988) has confirmed that not only do NFs differ from other IFs at both their amino- and carboxy-terminal regions but also in having a unique exon–intron organization. Moreover, the inferred amino-acid sequences of NF-M and NF-H both contain within their carboxy-terminal region the sequence Lys-Pro repeated 13 and 40 times, respectively. This repeat is in the region of the protein that is known to be highly phosphorylated (Julien and Mushynski, 1983) and thus may represent a kinase recognition site (Myers et al., 1987; Geisler et al., 1987; Lee et al., 1988).

To determine the biochemical and structural properties of NF proteins that are necessary for filament assembly, we have used DNA transfection to introduce either the NF-L or the NF-M genes of mouse into cultured fibroblast cells and examined the assembly properties of NF-L or NF-M expressed in this way.

Materials and Methods

Molecular Cloning of the Mouse NF-L and NF-M Genes

A recombinant mouse genomic DNA library in Charon 28 (obtained from Dr. P. Leder, Harvard University, Cambridge, MA) was screened by DNA hybridization with a 32P-labeled nucleotide sequence corresponding to the published cDNA sequence of NF-L (Lewis and Cowan, 1985). Four positive recombinants were obtained, of which one (as judged by restriction mapping and blot hybridization) contained the complete NF-L coding sequences including 5' and 3' end-flanking sequences (cf. Lewis and Cowan, 1986). The entire gene including the presumptive promoter sequences was isolated on a 7-kbp Hind III fragment and subcloned into pUC9 to produce pNF-L (see Fig. 1 A). DNA sequencing of fragments of pNF-L confirmed that the NF-L gene had been isolated, although a series of differences were found compared with the published mouse sequence (Lewis and Cowan, 1986).

To clone the NF-M gene, the mouse genomic library was screened with a 32P-labeled probe from cDNA clone HNF4, which encodes a portion of human NF-M (Myers et al., 1987). Three positive recombinants were obtained, two of which contained, by restriction mapping and hybridization, the complete mouse NF-M gene (cf. Levy et al., 1987). A 8-kbp Bam HI fragment containing the entire NF-M gene was isolated and subcloned into pUC19 to produce pNF-M (see Fig. 10 A).

Construction of pNF-L(Bam) and pMSV-NF-L

To ensure in transfection experiments that transcripts from pNF-L were distinguishable from endogenous NF-L RNAs, we introduced a smaller linker sequence into the 5' untranslated region of pNF-L. To do this, we subcloned the 1.8-kbp Hind III-Sma I fragment of pNF-L into the Hind III–Hind II sites of pUC8, and the large Mst II–Hind III fragment of pNF-L into the Hind II–Hind III site of pLUV. The resulting constructs were each double digested with Aat II and Bam HI (Aat II is present at a unique site in the pUC vector sequence of each construct) and the fragments containing NF gene sequences were ligated to produce the modified gene in pNF-L(Bam) (see Fig. 1 B). This procedure results in the net incorporation of 13 nucleotides between the abutting Sma I–Mst II sites of pNF-L (18 bases added, 5 deleted).

pMSV-NF-L (see Fig. 1 C) was constructed by replacing the Hind III–Bam HI fragment (containing the putative NF promoter sequences) of pNF-L(Bam) with a 0.6-kbp Hind III–Bgl II fragment that contains a Moloney Murome Sarcoma Virus (MSV) long terminal repeat (LTR) (isolated from pMSVTK- wt; kind gift of Dr. Steven L. McKnight, Carnegie Institution, Baltimore, MD) (Graves et al., 1985).

Construction of pMSV-NF-M

To obtain high level expression of NF-M in transfection assays, we constructed pMSV-NF-M (see Fig. 10 B) in which the F-0.9-kbp Bam HI–Stu I promoter sequences of pNF-M were replaced with the MSV LTR. To do this, we first digested pNF-M with Sac II and after removal of the F-0.9-kbp fragment religated the molecule (this procedure removes one of the two Stu I sites). The resulting molecule was double digested with Stu I and Bam HI and the large fragment containing the NF-M gene was ligated between the Xba I site (blunted with Klenow and deoxyribonucleotide triphosphates) and Bam HI site of a plasmid containing the MSV LTR. This plasmid was made by subcloning the 0.6-kbp Hind III–Xba I fragment of pMSV-NF-L (see Fig. 1 C; note that a Xba I site is located in the inserted polylinker common to pNF-L(Bam) and pMSV-NF-L) into the Hind III and Xba I sites of pUC19. To make a functional NF-M gene product, we finally reintegrated the Sac II fragment that had previously been removed) back in its correct orientation to produce pMSV-NF-M.

Primer Extension of RNA

The start point of transcription for the endogenous NF-L gene in mouse brain and for the transfected NF-L genes in cultured cells was determined by primer extension of RNA. Total RNA from adult mouse brain and tissue culture cells (24 h after transcription) was isolated by the guanidinium isothiocyanate/cesium chloride method (Chirgwin et al., 1979). The primer was the 72-bp Ava I–Ava I fragment (Ava I is an isoschizomer of the two Sma I sites of pNF-L) that had been 5' end-labeled with γ-32P[ATP and T4 polynucleotide kinase. Both radioactive strands were gel purified, and each strand was hybridized with 1 μg RNA (Casey and Davidson, 1977). cDNA was synthesized with reverse transcriptase (Life Sciences Inc., St. Petersburg, FL) and nonradioactive deoxyribonucleotide triphosphates. The products were recovered by ethanol precipitation and aliquots of each were electrophoresed on a 6% (wt/vol) polyacrylamide, 8 M urea gel calibrated with known size 32P-labeled Hpa II DNA filaments of pBR322. As expected, only one of the two primers (presumably the antisense strand) was extended into cDNA.

Tissue Culture and DNA Transfection

Mouse Ltk– cells, maintained in DME medium supplemented with 10% FCS, were transiently transfected with 10 μg DNA per 10-cm dish, using the DEAE-dextran method, followed 4 h later by a dimethyl sulphoxide shock (Lopata et al., 1984). Cells for immunofluorescence were translocated onto coverslips, or on dishes and then replated onto coverslips. Stable cell lines were obtained by transfecting mouse L cells (in 10-cm dishes) with 20 μg of a 10:1 mixture of pMSV-NF-L and pSV2–neo DNA using the calcium phosphate coprecipitation procedure (Graham and van der Eb, 1973) and selecting clones resistant to 400 μg/ml G418 (Gibco Labora-
Figure 1. Schematic drawings of the NF-L gene constructs and identification of the authentic NF-L transcription initiation site. (A) Schematic drawing of the 7-kb mouse genomic fragment from pNF-L that contains the NF-L gene. (B) Schematic drawing of the pNF-L(Bam) gene showing the 18-bp polylinker sequence inserted between the Sma I and Mst II sites of pNF-L (5 bases were also deleted leaving a net insertion of 13 bp). The Bam HI and Xba I sites in the linker sequence are underlined. (C) Schematic drawing of the MSV-NF-L gene in which the 1.8-kbp Hind III-Bam HI fragment of pNF-L(Bam) was replaced with the 0.6-kbp Hind III-Bgl II fragment that contains the Moloney MSV promoter segment (Graves et al., 1985). (D) 5' and 3' untranslated regions; (●) protein-coding sequences; (●) intron sequences. (D) Primer extension mapping of the start of transcription of NF-L RNAs in mouse brain and in cells transfected with the NF-L gene. The 72-bp Ava I-Ava I fragment of pNF-L (see Materials and Methods) was end labeled with $^{32}$P-ATP, the two radioactive strands were purified (a and b strands), hybridized with 1 µg RNA, and primer extended using reverse transcriptase. The radioactive products were separated by electrophoresis on a 6% polyacrylamide-urea gel and identified by autoradiography. Lane M, $^{32}$P-labeled Hpa II restriction fragments of pBR322; lanes 1 and 2, RNA from mock pUC18 transfected L cells; lanes 4 and 5, poly(A)+ RNA from mouse brain; lanes 7 and 8, RNA from L cells transfected with pNF-L(Bam) DNA; lanes 10 and 11, RNA from cells transfected with pMSV-NF-L DNA. Odd numbered lanes were analyzed with primer corresponding to the antisense mRNA strand; even numbered lanes are controls with the sense strand primer. Lanes 3, 6, 9, and 12 contain 1/10 of the amounts loaded in lanes 1, 4, 7, and 10, respectively.

Immunofluorescent Staining and Microscopy

L cells grown on glass coverslips were immersed for 30 s at 37°C in stabilization buffer (4 M glycerol, 100 mM Pipes, pH 6.9, and 1 mM EGTA), then extracted for 30 s at 37°C in stabilization buffer containing 0.5% (wt/vol) Triton X-100. The first wash was repeated, then the coverslips were plunged into −20°C chilled methanol for 5 min. After rehydration in standard PBS, the coverslips were incubated with antibody solution for 1 h at 37°C (antibodies were diluted in PBS containing 1% BSA). The coverslips were then washed in PBS and stained for 1 h at 37°C with fluorescent-conjugated antibodies. The coverslips were finally washed in PBS and mounted using Aqua-mount (Lerner Laboratories, New Haven, CT) on glass slides. Cells were examined on an Olympus BH-2 microscope using epifluorescence optics and photographed on Tri-X film (Eastman Kodak Co., Rochester, NY).

Primary antibodies used were mouse monoclonal antibodies to either NF-L, NF-M, or vimentin (Boehringer Mannheim Diagnostics, Inc., Houston, TX), a goat polyclonal antibody to vimentin (Polysciences Inc., War-lington, PA), and a rabbit polyclonal antibody to tubulin (Cleveland et al., 1981). Affinity-purified fluorescein and rhodamine-conjugated antibodies specific for each of the primary antibodies were obtained from Cappell Laboratories, Cochranville, PA.

SDS Gel Electrophoresis and Immunoblotting

Cells were harvested 2 d after transient transfection and lysed (except where otherwise stated) in 0.5% (wt/vol) SDS, 50 mM Tris, pH 6.8. Lysates were boiled in the presence of 2% β-mercaptoethanol and the proteins were separated by electrophoresis on 8.5% polyacrylamide gels (Laemmli, 1970). Proteins were transferred onto nitrocellulose filters (type BA83; Schleicher and Schuell, Inc., Keene, NH) and immunoblotted as described (Lopata and Cleveland, 1987). Cytoskeletal proteins were detected by autoradiography in the following manner: for NF-L, filters were first incubated with a mouse monoclonal anti-NF 68 antibody (Boehringer Mannheim Diagnostics, Inc.) followed by $^{125}$I-labeled goat anti-mouse antibody; for NF-M, a mouse monoclonal anti-NF 160 antibody (Boehringer Mannheim Diagnostics, Inc.) was used followed by $^{125}$I-labeled goat anti-mouse antibody; for vi-
Vimentin, a goat polyclonal anti-vimentin primary antibody was used followed by a rabbit anti-goat IgG and 125I-labeled Protein A, or a mouse monoclonal anti-vimentin antibody (Boehringer Mannheim Diagnostics, Inc.) followed by 125I-labeled goat anti-mouse antibody.

**Quantification of NF and Vimentin in Cell Lysates**

The amount of NF-L protein in stable cell lines was quantified by immunoblotting. For this analysis, the concentration of the 68-kD NF-L polypeptide in a crude preparation of mouse brain NFs (isolated by the axonal flotation procedure [Shelanski et al., 1971; Liem et al., 1978]) was first determined by comparing the intensity of Coomassie Blue stained SDS-polyacrylamide gel; (B) immunoblot of a parallel gel in which NF-L polypeptides were detected using an antibody specific for the NF-L polypeptide. Lane M, protein molecular mass markers (205, 116, 97, 66, 45, and 29 kD, respectively); lane J, protein extract from the dorsal root ganglia of rat; lane 2, proteins from L cells transfected with pMSV-NF-L DNA; lane 3, proteins from L cells transfected with pNF-L(Bam) DNA; lane 4, proteins from cells transfected with control pUC18 DNA. The arrowhead shows the presence of a 68-kD polypeptide in pMSV-NF-L transfected cells.

Results

**The NF-L Gene Is Transcribed When Introduced by Transfection into Mouse Fibroblasts**

To study the effects of inappropriate synthesis of NF-L in fibroblasts and to analyze the assembly properties of NF-L in this cell type, we initially cloned the mouse NF-L gene for use in DNA transfection experiments. A 7-kbp Hind III genomic fragment containing the entire mouse NF-L gene was isolated from a recombinant bacteriophage lambda clone, and subcloned into pUC9 to produce pNF-L (Fig. 1 A). To enable RNAs transcribed from pNF-L to be distinguished unambiguously from endogenous mouse NF-L transcripts, we inserted a linker sequence (net addition of 13 nucleotides) between the Sma I and Mst H sites that lie 24 and 31 bases, respectively, 5' to the AIG translation initiation codon (this yielded plasmid pNF-L[Bam]; Fig. 1 B, see Materials and Methods for details of the construction). Since RNAs from the endogenous NF-L gene accumulate only in neuronal cells (presumably the consequence of transcriptional control), we were concerned that the NF-L gene might not be expressed or be expressed only at low levels after transfection into nonneuronal cells. Consequently, we also constructed plasmid pMSV-NF-L (see Fig. 1 C) in which the strong promoter from the MSV LTR (Laimins et al., 1982; Graves et al., 1985; Izant and Weintraub, 1984) was substituted for the presumptive NF-L promoter segment. To do this, we replaced the 1.8-kbp Hind III–Bam HI fragment of pNF-L(Bam) with a 0.6-kbp Hind III–Bgl II fragment containing the MSV LTR.

pNF-L(Bam) and pMSV-NF-L were transiently transfected into mouse L cells and NF-L RNAs were identified by primer extension. Parallel analysis of mouse brain RNA (Fig. 1 D, lane 4) identified the authentic transcription initiation site in neurons to lie 173 bases 5' to the primer (104 bases 5' to the translation initiation codon). SI nuclease protection experiments (not shown) confirmed this identification (which had not been previously mapped). Surprisingly, abundant RNAs also initiated at this site (after taking into account the 13 nucleotide linker inserted into pNF-L[Bam]) in the fibroblasts transfected with pNF-L(Bam) (Fig. 1 D, lane 7). Even more abundant RNAs (10–20-fold) derived from the MSV-promoted pMSV-NF-L-transfected DNA (Fig. 1 D, lane 10) were found. As expected, no endogenous NF-L transcripts were detectable in L cells (Fig. 1 D, lane 1).
NF-L polypeptides synthesized in transfected L cells are found exclusively in the cytoskeleton. 48 h after transfection with plasmid DNAs, mouse L cells were harvested, lysed (in 0.5% [wt/vol] Triton X-100, 4 M glycerol, 1 mM EGTA, 100 mM Pipes, pH 6.9, at 20°C), and centrifuged at 10,000 rpm for 10 min. Equal proportions of the supernatant (lanes d, f, and h), and pellet (cytoskeleton; lanes c, e, and g) were electrophoresed on a SDS-polyacrylamide gel. The presence of NF-L polypeptides was then determined by immunoblotting with the NF-L specific antibody. Lane a, protein molecular mass markers (205, 116, 97, 66, 45, and 29 kD, respectively); lane b, protein extract from the dorsal root ganglia of rat; lanes c–h, proteins from L cells transfected with control pUC18 DNA (lanes c and d), pNF-L(Bam) DNA (lanes e and f), or pMSV-NF-L DNA (lanes g and h). (A) Coomassie Blue-stained gel; (B) immunoblot.

Figure 3.

**Figure 4.** Immunofluorescence detection of NF-L in transfected L cells. Mouse L cells grown on coverslips were transfected with pMSV-NF-L DNA, extracted in Triton/glycerol buffer, and stained for NF-L using the mouse monoclonal NF-L antibody followed by fluorescein-conjugated rabbit anti-mouse IgG. (A) Mock-transfected cells; (B–D) examples of cells showing NF staining after transfection with pMSV-NF-L DNA. Bar, 10 μm.
Monteiro and Cleveland Assembly of NF-L and NF-M in Nonneuronal Cells 585

Figure 6. Localization of IFs and tubulin in transfected cells by double-label immunofluorescence. 48 h after transfection with pMSV-NF-L DNA, cells were double stained either for vimentin and tubulin (A and B) or for NF-L and tubulin (C and D). Cells were extracted in Triton/glycerol stabilization buffer (see Materials and Methods) and fixed. To detect vimentin and tubulin a goat polyclonal anti-vimentin antibody and a mouse monoclonal anti-α-tubulin antibody (Amersham Corp., Arlington Heights, IL) were used followed by a fluorescein-conjugated rabbit anti-goat IgG and a rhodamine-conjugated rabbit anti-mouse IgG. For localization of NF-L and tubulin, NF-L was detected with the mouse monoclonal anti-NF-L antibody and tubulin detected with a rabbit polyclonal antitubulin antibody (Cleveland et al., 1981) followed by sheep fluorescein-conjugated anti-mouse IgG and a rhodamine-conjugated goat anti-rabbit IgG. Bar, 10 μm.

The NF-L Polypeptide Is Assembled into Cytoskeletal IF Arrays in Fibroblasts

To determine if NF-L protein was synthesized and accumulated in cells transfected with pNF-L(Bam), cell extracts were analyzed for NF-L polypeptide accumulation by protein blotting using an NF-L-specific monoclonal antibody. Immunoblots of protein extracts showed the presence of a low level of an ~68-kD protein in cells transfected with pNF-L(Bam) that was not present in mock-transfected cells (Fig. 2 B, compare lanes 3 and 4). Further, this protein was indistinguishable in size from the authentic NF-L protein found in mouse or rat brain extracts (Fig. 2 B, lane 1). Moreover, pMSV-NF-L-transfected cells contained sufficiently elevated levels of an immunoreactive 68-kD polypeptide (Fig. 2 B, lane 2) that the polypeptide was identifiable by Coomassie Blue staining (see arrowhead in Fig. 2 A, lane 2).

We next examined whether NF-L polypeptides synthesized in transiently transfected fibroblasts were assembled into IFs

Figure 5. Localization of NF and vimentin in transfected cells using double-label immunofluorescence. (A-F) 48 h after transfection with pMSV-NF-L DNA, cells were stained with the mouse monoclonal NF-L antibody and a goat polyclonal vimentin antibody followed by fluorescein-conjugated rabbit anti-mouse IgG and rhodamine-conjugated rabbit anti-goat IgG. Fluorescein images of NF-L (A, C, and E); rhodamine images of the vimentin pattern in the same cells (B, D, and F). (G and H) Transfected cells were treated 48 h after transfection with 1 μM Colcemid for an additional 18 h, and then coverslips were examined (G) for NF-L localization or (H) for vimentin. Bar, 10 μm.
and if so whether they formed a new array separate from the endogenous vimentin array or alternatively whether NFs coassembled with vimentin. As an initial experiment we determined whether the NF-L polypeptides were found in the insoluble cytoskeletal fraction. For this, a soluble and a cytoskeleton pellet fraction were prepared from cells 48 h after transfection with either pNF-L(Bam) DNA or with pMSV-NF-L DNA (see Materials and Methods). Immunoblotting was used to analyze an equal proportion of each fraction for the presence of NF-L protein. As shown in Fig. 3, the NF-L protein was found exclusively in the insoluble, cytoskeletal fraction.

The assembly products of NF-L in the transiently transfected fibroblasts were localized using immunofluorescence. L cells plated onto glass coverslips were transfected with either pMSV-NF-L or mock transfected with pUC18 DNA and after 21 or 48 h were extracted in buffer containing 0.5% Triton X-100 (see Materials and Methods). The cells were then incubated with the monoclonal anti-NF-L antibody followed by a rabbit anti–mouse secondary antibody labeled with fluorescein. As expected, visualization of cells transfected with pUC18 DNA contained no fluorescent-staining material (Fig. 4 A). However, as early as 24 h after transfection, ~30% of the cells transfected with pMSV-NF-L DNA stained positive for NF-L (see Fig. 4 B). By 48 h, the total number of cells that contained NF-L staining increased to ~50%, although the qualitative appearance of the staining varied markedly from cell to cell. Fig. 4 shows examples of cells that had such divergent staining patterns. Most (70%) positively staining cells contained filaments that extended throughout the cytoplasm (Fig. 4, B–D). The filaments were radial and more abundant around the nucleus, a distribution typical for IFs in cultured cells (Franke et al., 1978, 1979). In contrast, some cells contained a very unusual and heterogeneous distribution of NF-L. For example, Fig. 4 D shows a cell in which NF-L accumulated at the extreme ends of projections of the cell and appeared discontinuous not only from one another but also from a large area of NF-L accumulation in the center of the cell. The latter densely staining, spherical mass may represent aggregation or precipitation of NF-L into an amorphous structure (Fig. 4 D). Aggregates like these usually were found in close proximity to the nucleus. In some extreme cases the whole cell appeared to be filled with NF-staining material (Fig. 4 C, arrow).

**NF-L Expressed in Fibroblasts Forms Copolymers with Endogenous Vimentin**

To test whether expression of NF-L in mouse fibroblasts would affect the existing array of vimentin-containing filaments and whether subunits would coassemble in vivo, we colocalized vimentin and NF-L by using double-immunofluorescence microscopy. 48 h after transfection of L cells with pMSV-NF-L DNA, NF-L was visualized in the fluorescein channel (Fig. 5, A, C, E, and G) and vimentin localized in rhodamine fluorescence (Fig. 5, B, D, F, and H). All the cells contained a characteristic network of anti–vimentin staining, whereas only ~50% stained with anti–NF-L. At the resolution of the light microscope, it was nevertheless evident that usually the anti–NF-L fluorescence pattern represented a subset of the anti–vimentin fluorescence. In no case could we trace an individual filament that stained for NF-L but not with vimentin. In fact, all filaments that could be followed for both NF-L and vimentin were superimposable on one another.

To investigate further this apparent copolymerization, we examined the effects of microtubule depolymerization on the NF and vimentin arrays. A classical characteristic of IFs is that after drug-induced microtubule depolymerization the IFs aggregate and form perinuclear whorls (e.g., Ishikawa et al., 1968; Goldman, 1971). This proved also the case for NF-L arrays in transfected cells. When treated for 18 h with 1 μM Colcemid (Sigma Chemical Co., St. Louis, MO) to induce microtubule disassembly and examined for NF-L localization, transfected cells displayed the characteristic collapse of both NF-L (Fig. 5 G) and vimentin-staining (Fig. 5 H) filaments.

**Spatial Distribution of IFs and Microtubules in Transfected Cells**

Previous studies of double-immunofluorescence patterns of IFs (desmin or vimentin) and of microtubules have suggested that (at least in some cells) there is a high degree of correlation or coincidence in the organization of these two cytoskeleton components (e.g., Geiger and Singer, 1980; Singer et al., 1982). To determine if this was so in L cells, we performed a similar double-immunofluorescence experiment for vimentin and tubulin staining. As shown in Fig. 6, A and B, there was indeed a high coincidence of alignment between the vimentin and tubulin filaments in mouse L cells. We next examined the coincidence of NF-L- and tubulin-staining filaments after transfection of L cells with pMSV-NF-L DNA. Because NF-L staining was so widespread within the cytoplasm, and especially around the nucleus, it was difficult to trace the path of individual filaments. Except at the cell periphery, where individual filaments could be tracked more easily, it was possible only to compare the gross distribution of the filaments in the whole cell. Nonetheless, not only was the overall distribution of the two proteins strikingly similar in all cells (Fig. 6, compare C with D), many NF-L- and tubulin-containing filaments colocalized (although there were many other examples where one filament type could not be superimposed on the other type).

**Isolation of Stable L Cell Lines Expressing NF-L**

To analyze whether accumulation of NF-L polypeptides into vimentin arrays would have any observable effect on cell viability or growth characteristics, transient transfection was unsuitable. Consequently, we produced cell lines in which pMSV-NF-L DNA was stably integrated and expressed. For this, we cotransfected 2 × 10⁶ mouse L cells with pMSV-NF-L and pSV2–neo DNA and obtained 48 stable cell lines that were resistant to G418. Five out of 14 cell lines (MSV-NF2, MSV-NF8, MSV–NF9, MSV–NF10, and MSV–NF13) that were analyzed for NF-L expression contained a 68-kD polypeptide that reacted with the NF-L-specific antibody on immunoblots (Fig. 7 B). The two lines expressing the highest levels of NF-L (MSV-NF2 and MSV–NF9) were studied in more detail. Careful quantitative immunoblotting with known amounts of mouse brain NF-L as a standard revealed that the steady-state level of NF-L synthesized in MSV–NF2 and MSV–NF9 cells was 0.5 ± 0.05 and 8.9 ± 0.2% of total cell protein (see Fig. 7 C and Materials and Methods). Steady-state vimentin represented 1.3 ± 0.1% of total pro-
Figure 7. Accumulation of NF-L polypeptides in cell lines stably transfected with pMSV-NF-L. (A) Coomassie Blue–stained gel of total protein lysates (30 μg per lane) of G418-resistant cell lines cotransfected with pMSV-NF-L and pSV2-neo DNA; (B) immunoblot detection of NF-L. (Note that in cell line MSV-NF9, the 68-kD NF-L polypeptide can be clearly seen in the Coomassie Blue–stained gel [lane d] with an intensity approximately the same as the 43-kD actin band.) (C) Quantification of NF-L produced in stable cell line MSV-NF2 relative to known amounts of mouse brain NF-L polypeptide. NF-L was purified from mouse brain and known quantities (shown in ng, lanes a–i, and determined relative to known amounts of serum albumin by Coomassie Blue staining) was electrophoresed together with 15 and 7.5 μg of total protein from cell line MSV-NF2 (lanes j and k). The proteins were transferred onto a nitrocellulose filter and immunoblotted for NF-L. (D) Quantification of vimentin produced in cell lines MSV-NF2 and MSV-NF9. Vimentin levels were quantified similarly to that used for NF-L except that known amounts of vimentin (shown in ng; lanes a to g) in crude L cell cytoskeletons were used as the standard. The amount of vimentin in 5 and 10 μg of total L cell protein lysate (lanes h and i, respectively) and 10 μg of protein lysate in the two cell lines MSV-NF9 and MSV-NF2 (lanes j and k, respectively) was determined by scanning the bands in the autoradiogram shown.
tein, an amount similar to that found in untransfected L cells (compare lanes h–k in Fig. 7 D).

All MSV-NF2 and MSV-NF9 cells stained positive for NF-L (as expected) by indirect immunofluorescence microscopy (Fig. 8. A and B, respectively). The cells contained a filamentous-like pattern of NF-L staining, typical of the arrangement of IFs in fibroblasts. (Cells transiently transfected are bigger than those from stable transfections. This is not due to expression of NF-L, but rather is a DNA-independent consequence of the transient transfection procedure.) The staining pattern was different in the two cell lines. Many MSV-NF9 cells contained aggregates of NF-L material, but only a few MSV-NF2 cells contained such aggregates. This probably was the result of higher expression of NF-L in the MSV-NF9 cell line. However, the level of NF-L accumulation in both cell lines did not appear to be as high as in some transiently transfected cells, when a mass of NF-L material was found to completely fill the cytoplasm (compare the cell arrowed in Fig. 4 C with those in Fig. 8 B).

**Coassembly with NF-L Changes the Solubility Characteristics of the Endogenous Vimentin Filaments**

To examine coassembly of NF-L and vimentin subunits in another way, we tested whether filaments composed of the two different subunits could be distinguished biochemically by an alteration in the solubility properties expected for homopolymers assembled from either subunit. Thus, we examined IFs in mock-transfected L cells (which should contain IFs that are homopolymers of vimentin), in L cells transiently transfected with pMSV-NF-L (which could contain IFs that are homopolymers and/or heteropolymers of vimentin and NF-L subunits), and in cell lines MSV-NF2 and MSV-NF9 (which could contain IFs that are homopolymers and/or heteropolymers of vimentin and NF-L). Cell aliquots were extracted under a variety of different conditions and an equal fraction of each extract was separated by SDS-PAGE (Fig. 9 A). Immunoblot analyses of parallel gels probed for vimentin (Fig. 9 C) and NF-L (Fig. 9 B) showed a clear increase in the proportion of vimentin in the later extraction steps of cells expressing NF-L. For example, compared to mock-transfected cells, almost as much vimentin remained in the final pellet fraction of NF-L-expressing cells. NF-L solubility also appeared to be affected by its abundance relative to vimentin. In line MSV-NF2 that contains NF-L and vimentin in a 1:2.6 molar ratio, NF-L was the most soluble, with a substantial amount cosolubilizing in a fraction enriched in vimentin (Fig. 9, B and C, lanes b). In line MSV-NF9 (in which NF-L/vimentin is 7:1), a smaller but still significant amount of NF-L solubilized in lane b. Least soluble was NF-L expressed transiently. Since in transient experiments the level of expression is highly variable from cell to cell, NF-L stoichiometry to vimentin is also highly variable, but on average is probably higher than in stable lines expressing MSV-NF-L. (That only a modest difference is observed for vimentin solubility in transiently transfected cell populations is expected since a majority of the cells [the ones not successfully transfected] express no NF-L.)

**Cloning and Expression of the Mouse NF-M Gene: Apparent Coassembly of NF-M with Vimentin**

In view of the assembly of NF-L in fibroblasts, we were interested in determining whether NF-M expressed in these cells was also competent to form filaments. Thus, we isolated the mouse NF-M gene (see Materials and Methods) and an 8-kbp Bam HI fragment containing the entire NF-M gene was subcloned into pUC19 to produce pNF-M (Fig. 10 A). We also made a construct, pMSV-NF-M, in which the presumptive NF-M gene promoter was substituted with the MSV LTR (see Fig. 10 B and Materials and Methods).

Plasmids pNF-M and pMSV-NF-M were transiently transfected into L cells and 48 h after transfection the proteins were separated by electrophoresis and immunoblotted for the presence of NF-M (Fig. 10, C and D) using a monoclonal antibody specific for NF-M (Boehringer Mannheim Diagnostics, Inc.). An ~130-kD protein that reacted with the NF-M antibody was found in cells transfected with pNF-M DNA (Fig. 10, C and D, lanes 2) but was not found in cells mock transfected with pUC19 DNA (Fig. 10, C and D, lanes 1). This protein accumulated to a level at least 10-fold higher in cells transfected with pMSV-NF-M DNA (Fig. 10, C and D, lanes 3).

Localization of NF-M by immunofluorescence light microscopy revealed that ~20% of cells transfected with pMSV-

![Figure 8. Immunofluorescence localization of NF-L polypeptides in cells stably transfected with pMSV-NF-L. (A) Cell line MSV-NF2; (B) cell line MSV-NF9. Staining was performed as detailed in the legend to Fig. 4. Bar, 10 μm.](image-url)
Figure 9. Differential solubility of vimentin and NF-L in transiently transfected cells and stable cell lines expressing NF-L. Mouse L cells either mock transfected with pUC19 DNA or pMSV-NF-L DNA and two cell lines, MSV-NF2 and MSV-NF9, expressing NF-L were extracted under different conditions (see Materials and Methods) and the proteins were separated by electrophoresis. (A) Coomassie Blue-stained SDS-polyacrylamide gel. The position of NF-L and vimentin are shown on the right hand side in lane e (N and V, respectively). Lane a, Triton X-100-soluble proteins; lane b, proteins solubilized at 4°C; lane c, proteins solubilized in 0.6 M KCl at 20°C; lane d, proteins solubilized in 1 M urea; lane e, proteins solubilized in 2 M urea; and lane f, the residual pellet fraction. (B) Immunoblot of a parallel gel probed for NF-L (below which is shown a histogram of the distribution of NF-L polypeptides in each fraction [as determined by scanning the immunoblots]). (C) Immunoblot of a parallel gel and histogram probed for vimentin.
Figure 10. Schematic drawings of the NF-M gene constructs, immunoblot, and immunofluorescence detection after NF-M transfection into fibroblasts. (A) Schematic drawing of the 8-kbp Bam HI mouse genomic fragment of pNF-M that contains the NF-M gene. (B) Schematic drawing of pMSV-NF-M in which the ~0.9-kbp Hind III–Stu I fragment of pNF-M was replaced with a 0.6-kbp Hind III–Xba I fragment containing the MSV LTR (see Materials and Methods). (A and B) (m) 5' and 3' untranslated regions; (m) protein-coding sequences; (m) intron sequences. (C and D) Immunoblot detection of NF-M polypeptides after transfection of the NF-M gene into mouse L cells. Mouse L cells were harvested 48 h after transfection, lysed, and analyzed for the presence of NF-M polypeptides by immunoblotting. (C) Coomassie Blue-stained gel; (D) immunoblot. Lane M, protein molecular mass markers (205, 116, 97, 66, 45, and 29 kD, respectively); lane 1, proteins from L cells transfected with pUC19 DNA; lane 2, proteins from cells transfected with pNF-M DNA; and lane 3, proteins from cells transfected with pMSV-NF-M DNA. (E and F) Immunofluorescence detection of NF-M in transfected L cells. Mouse L cells grown on coverslips were transfected with pMSV-NF-M DNA, and stained for NF-M filaments using the mouse monoclonal NF-M antibody essentially as described in Fig. 4. (E) Cells mock transfected with pUC19 DNA; (F) cells transfected with pMSV-NF-M DNA. (G and H) Double immunofluorescence localization of cells transfected with pMSV-NF-M DNA. (G) Staining with anti-NF-M antibodies; (H) staining for vimentin. Bar, 10 μm.
NF-M DNA contained IF-like arrays that stained with the antibody (Fig. 10 F). In fact, NF-M-staining material in almost all of the cells was assembled into filaments, and few if any aggregates were seen, thus demonstrating the competence of NF-M to form filaments in vivo. (Mock-transfected cells were unstained [Fig. 10 E].) Simultaneous staining of cells with both vimentin and NF-M antibodies revealed the colocalization of filaments containing NF-M (Fig. 10 G) and vimentin (Fig. 10 H). This apparent coassembly in transfected cells was obvious in many filaments, although many others (particularly those near the cell periphery) stained only with vimentin.

Discussion

To examine the factors that govern NF assembly in vivo, we have characterized the consequences of inappropriate expression of NF-L and NF-M subunits in fibroblast cells. Although NF-L and NF-M are normally restricted in their expression to postmitotic neurons, we have found that when expressed in fibroblasts they are incorporated into IF-like arrays. With the caveat that we cannot rule out the possibility that expression of NF subunits in fibroblasts resulted in the induction of proteins normally associated with their expression in neurons, these data lead us to conclude that expression of a single NF-L or NF-M protein, without the need for any accessory neuronal-specific proteins, is sufficient for forming filaments in a fibroblast cell. (While such putative inductions may at first seem implausible, one example was recently documented for the induction of a type I keratin in fibroblasts after transfection of a type II keratin gene [Guidice and Fuchs, 1987].) Mindful of the above cautions, the high coincidence in the spatial distribution of NF-L filaments and microtubules in the transfected cells also suggests both that there is a close and dynamic interplay in the organization of NFs and microtubules in forming the cellular cytoskeleton and that this interaction does not require additional neuronal-specific components.

While assembly of NF-L polypeptides into filaments might be the expected result in light of their competence for self-assembly in vitro (Geisler and Weber, 1981), two lines of evidence showed that the majority of assembled NF-L filaments are comprised of copolymers with the endogenous vimentin polypeptides. First, by double immunofluorescence microscopy of the transfected cells, we found the NF-L-staining pattern to be superimposable on the vimentin-staining pattern. In fact, in the stable cell lines expressing NF-L, the vimentin pattern was also indistinguishable from the NF-L-staining pattern (data not shown). Second, accumulation of NF-L polypeptides not only decreased the solubility of the endogenous vimentin filaments (Fig. 9 C), but also slightly increased the solubility of NF-L (Fig. 9 B). This provides further evidence that filaments containing both subunits must be present. A similar effect in which vimentin and desmin heteropolymers had solubility properties intermediate between those of the homopolymer IFs had been previously documented (Steinert et al., 1981).

The copolymerization of NF-L and vimentin in vivo is perhaps not too surprising, in as much as the two proteins both contain the highly conserved ~310 amino acid rod segment that has been shown to be essential for filament formation. This rod segment is also highly conserved in desmin and GFAP, both of which also form copolymers with vimentin (Quinlan and Franke, 1982, 1983; Quax et al., 1985; and Wang et al., 1984, respectively). Unlike desmin, vimentin, and GFAP (all of which contain a single cysteine residue in the 'd' position of the heptad repeat element of the rod segment), in NF-L the cysteine is in the 'd' position. Since it now appears that the first step in IF assembly is probably the formation of a coiled–coil dimer in a parallel, in register fashion which then associates with another dimer to produce a tetramer probably in an antiparallel arrangement in the final filament (e.g., Geisler and Weber, 1986; Quinlan et al., 1986), the role of the single cysteine remains unclear. Using cross-linking studies, it has been shown (Quinlan and Franke, 1982, 1983; Quinlan et al., 1986) that the two cysteines in the two parallel chains of a dimer form an intramolecular cross-link. However, it is well known that disulfide bridge formation is not essential for maintenance of IF structure and assembly since both these processes take place in the presence of high concentrations of reducing agents (Steinert et al., 1976, 1981; Rueger et al., 1979; Renner et al., 1981; Geisler and Weber, 1981). From the present study, we do not know whether the heteropolymers of NF-L and vimentin we have observed by immunofluorescence are composed of either vimentin/vimentin, NF-L/NF-L, or NF-L/vimentin dimers. If the cysteine does have a role in IF assembly, and in view of the difference in its location in NF-L and vimentin, it would seem more likely that the heteropolymers arise due to assembly of vimentin/vimentin dimers with NF-L/NF-L dimers.

The incorporation of NF-M into filaments (and partial colocalization with vimentin) was more unexpected but it is consistent with previous in vitro studies that concluded that under certain conditions NF-M would self-assemble to form filaments (see Introduction). It is now clear from our data that NF-M will form filaments in vivo without the apparent need for other neural-specific proteins (in particular NF-L, which is known to facilitate NF-M assembly).

The reason why aggregates of NF-L, in addition to filaments, were found in some transiently transfected cells is not certain. However, since these were present only in the most brightly staining cells, it seems most likely that a high level of NF expression results in its precipitation, as is frequently found when foreign proteins are expressed in the prokaryotes, Escherichia coli and Bacillus subtilis. It should be noted that similar aggregates were seen when a hamster desmin gene and a type K14 keratin were expressed in heterologous systems (Quax et al., 1985; Guidice and Fuchs, 1987).

It is curious that L cells transfected with the NF-L gene carrying the authentic NF promoter region transcribed the plasmid-borne gene and did so from the same transcription initiation site (104 nucleotides upstream of the ATG translation initiation codon) as is used in authentic neurons. Why the endogenous NF-L genes in mouse L cells are not transcribed is not known, but it is clear that it cannot be due to the absence of a required transcription factor. Presumably, the chromosomal genes contain trans-acting elements that prevent their transcription by blocking access of necessary transcription factors or the local chromosomal structure has been altered to prevent activation.

Finally, the successful isolation of stable cell lines that express NF-L as the second most abundant cell protein (as much as 9% of cell protein, a level approximately sevenfold that of vimentin), demonstrates that expression and incorpo-
ration of at least this level of NF-L into filaments does not interfere with the normal cellular growth processes of mitosis and cell division. In fact, no differences in growth rates or cell morphology are apparent in these cell lines. Moreover, the stable line that expresses NF-L as 9% of cell protein has been cultured continuously for over 6 mo. No diminution of NF-L accumulation has been detected and >99% of the cells continue to stain positively (data not shown). Since NF-L is normally expressed exclusively in postmitotic neurons, that high levels of highly insoluble NF-L filaments are tolerated in rapidly dividing cells may be a bit surprising. It will be interesting to see these stable lines in future experiments to follow the assembly/disassembly of NF-L filaments during the cell cycle. These lines also offer the potential for introduction of additional NF-M and NF-H genes so as to elucidate the interactions that occur during filament formation.

We thank Chee Chun for isolating NF-L clones and Margaret Lopata for providing the tubulin antibody and for advice on immunofluorescence microscopy. We also thank Dr. Sangram Sisodia for help in producing stable transfectants.

This work has been supported by grants from the March of Dimes and the American Heart Association to D. W. C. Cleveland. M. J. Monteiro is a postdoctoral fellow of the Maryland Affiliate of the American Heart Association.

Received for publication 18 August 1988, and in revised form 6 October 1988.

References

Albers, K., and E. Fuchs. 1987. The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. J. Cell Biol. 105:791-806.

Carden, M. J., W. W. Schiaffer, and V. M.-Y. Lee. 1985. The structure, biochemical properties and immunogenecity of neurofilament peripheral regions are determined by phosphorylation state. J. Biol. Chem. 260:9805-9817.

Casey, J., and N. Davidson. 1977. Rates of formation and thermal stabilities of RNA:DNA:DNA duplexes at high concentrations of formaldehyde. Nucleic Acids Res. 4:1539-1552.

Chirgwin, J. M., A. E. Przybyla, R. J. Macdonald, and W. J. Rutter. 1979. Isolation of biologically active ribonuclease from sources enriched in ribonuclease. Biocem. Biophys. Res. Commun. 101:526-530.

Cleveland, D. W., M. A. Lopata, P. Sherline, and M. W. Kirschner. 1981. Unpolymerized tubulin modulates the level of tubulin mRNA. Cell. 25:537-546.

Frank, W. W. 1987. Nuclear lamins and cytoskeletal intermediate filament proteins: a growing multigene family. Cell. 48:3-4.

Franke, W. W., E. Schmid, M. Osborn, and K. Weber. 1978. Different intermediate-size filaments distinguished by immunofluorescence microscopy. Proc. Natl. Acad. Sci. U.S.A. 75:5034-5038.

Franke, W. W., E. Schmid, S. Winter, M. Osborn, and K. Weber. 1979. Widespread occurrence of intermediate-sized filaments of the vimentin-type in cultured cells from diverse vertebrates. Exp. Cell Res. 123:25-46.

Gardner, E. E., D. Dahl, and A. Bignami. 1984. Formation of 10-nanometer filaments from the 150-k dalton neurofilament protein in vitro. J. Neurosci. Res. 11:145-155.

Geiger, B., and S. J. Singer. 1980. Association of microtubules and intermediate filaments in BHK-21 cells as detected by double immunofluorescence. Proc. Natl. Acad. Sci. U.S.A. 77:4769-4773.

Geisler, N., and K. Weber. 1982. The amino acid sequence of chicken muscle protein. J. Biol. Chem. 257:10467-10470.

Geisler, N., and K. Weber. 1986. Structural aspects of intermediate filaments in developing skeletal muscle. J. Cell Biol. 96:538-555.

Geisler, N., and K. Weber. 1987. Location and sequence characterization of the major phosphorylation sites of the high molecular mass neurofilament proteins M and H. FEBS (Fed. Eur. Biochem. Soc.) Lett. 221:403-407.

Giudici, O. J., and E. Fuchs. 1987. The transfection of epidermal keratin genes into fibroblasts and simple epithelial cells: evidence for inducing a type I keratin by a type II gene. Cell. 48:453-463.

Goldman, R. D. 1971. The role of three cytoplasmic fibres in BHK-21 cell motility. I. Microtubules and the effects of colchicine. J. Cell Biol. 51:752-762.

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.

Graves, B. J., R. N. Eisenman, and S. L. McKnight. 1985. Deliberation of transcriptional control signals within the moleone murine sarcoma virus long terminal repeat. Mol. Cell. Biol. 5:1948-1958.

Hirakawa, N., M. A. Glicksman, and M. B. Willard. 1984. Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton. J. Cell Biol. 98:1523-1536.

Ishikawa, H., R. Bischoff, and H. Hotzler. 1968. Mitosis and intermediate-size filaments in developing skeletal muscle. J. Cell Biol. 36:525-546.

Johansen, D. E., D. Dahl, and A. Bignami. 1984. Formation of 10-nanometer filaments in rapidly dividing cells may be a bit surprising. It will be interesting to see these stable lines in future experiments to follow the assembly/disassembly of NF-L filaments during the cell cycle. These lines also offer the potential for introduction of additional NF-M and NF-H genes so as to elucidate the interactions that occur during filament formation.

We thank Chee Chun for isolating NF-L clones and Margaret Lopata for providing the tubulin antibody and for advice on immunofluorescence microscopy. We also thank Dr. Sangram Sisodia for help in producing stable transfectants.

This work has been supported by grants from the March of Dimes and the American Heart Association to D. W. C. Cleveland. M. J. Monteiro is a postdoctoral fellow of the Maryland Affiliate of the American Heart Association.

Received for publication 18 August 1988, and in revised form 6 October 1988.
Quinlan, R. A., and W. W. Franke. 1982. Heteropolymer filaments of vimentin and desmin in vascular smooth muscle tissue and cultured baby hamster kidney cells demonstrated by chemical crosslinking. Proc. Natl. Acad. Sci. USA. 79:3452–3456.

Quinlan, R. A., and W. W. Franke. 1983. Molecular interactions in intermediate-sized filaments revealed by chemical cross-linking. Heteropolymers of vimentin and glial filament protein in cultured human glioma cells. Eur. J. Biochem. 132:477–484.

Quinlan, R. A., M. Hatzfeld, W. W. Franke, A. Lustig, T. Schultbess, and J. Engel. 1986. Characterization of dimer subunits of intermediate filament proteins. J. Mol. Biol. 192:337–349.

Renner, W., W. W. Franke, E. Schmid, N. Geisler, K. Weber, and E. Mandelkow. 1981. Reconstitution of intermediate-sized filaments from denatured monomeric vimentin. J. Mol. Biol. 149:285–306.

Rueger, D. C., J. S. Huston, D. Dahl, and A. Bignami. 1979. Formation of 100Å filaments from purified glial fibrillary acidic proteins in vitro. J. Mol. Biol. 135:53–68.

Sharp, G. A., G. Shaw, and K. Weber. 1982. Immunoelectron microscopical localization of the three neurofilament triplet proteins along neurofilaments of cultured dorsal root ganglion neurons. Exp. Cell Res. 137:403–413.

Shelanski, M. L., S. Albert, G. H. DeVries, and W. T. Norton. 1971. Isolation of filaments from brain. Science (Wash. DC). 174:1242–1245.

Singer, S. J., E. H. Ball, B. Geiger, and W.-T. Chen. 1982. Immunolabeling studies of cytoskeletal associations in cultured cells. Cold Spring Harbor Symp. Quant. Biol. 46:303–316.

Smith, P. K., R. I. Kroeha, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. H. Olson, and D. C. Klenk. 1985. Measurement of protein using bichinchoninic acid. Anal. Biochem. 150:76–85.

Steinert, P. M., W. W. Idler, F. Cabral, M. M. Gottesman, and R. D. Goldman. 1981. In vitro assembly of homopolymer and copolymer filaments from intermediate filament subunits of muscle and fibroblastic cells. Proc. Natl. Acad. Sci. USA. 78:3692–3696.

Steinert, P. M., W. W. Idler, and S. B. Zimmerman. 1976. Self-assembly of bovine epidermal keratin filaments in vitro. J. Mol. Biol. 108:547–567.

Steinert, P. M., R. H. Rice, D. R. Roop, B. L. Trus, and A. C. Steven. 1983. Complete amino acid sequence of a mouse epidermal keratin subunit and implications for the structure of intermediate filaments. Nature (Lond.). 302:794–800.

Sternberger, L. A., and N. M. Sternberger. 1983. Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. Proc. Natl. Acad. Sci. USA. 80:6126–6130.

Tokutake, S., R. K. H. Liem, and M. L. Shelanski. 1984. Each component of neurofilament assembles itself to make component-specific filament. Biochem. Res. 5:235–238.

Traub, P., and C. E. Vorgias. 1983. Involvement of the N-terminal polypeptide of vimentin in the formation of intermediate filaments. J. Cell Sci. 63:43–67.

van den Heuvel, R. M. M., S. Albert, C. J. M. van Eys, F. C. S. Ramaekers, W. J. Quax, W. T. M. Vree Egberts, G. Schaart, T. M. Cuypers, and H. Bloemendaal. 1987. Intermediate filament formation after transfection with modified hamster vimentin genes. J. Cell Sci. 88:475–482.

Wang, E., J. G. Curnier, and R. K. H. Liem. 1984. Identification of glial filament protein and vimentin in the same intermediate filament system in human glioma cells. Proc. Natl. Acad. Sci. USA. 81:2102–2106.