Determinants for Intracellular Sorting of Cytoplasmic and Nuclear Intermediate Filaments

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Abstract. The mechanism by which nuclear and cytoplasmic filaments are sorted in vivo was studied by examining which lamin sequences are required to target an otherwise cytoplasmic IF protein, the small neurofilament subunit (NF-L), to the nuclear lamina. By swapping corresponding domains between NF-L and lamin A, nuclear envelope targeting of NF-L was shown to require the presence of the "head" domain, a 42–amino acid sequence unique to lamin rod domains, a nuclear localization signal and the CAAX motif. Replacement of the entire COOH-terminal tail of lamin A with that of NF-L had no discernible effect on nuclear localization of lamin A, provided the substituted NF-L tail contained a NLS and a CAAX motif. This chimeric protein exhibited characteristics more typical of lamin B than that of the parental lamin A.

With regard to cytoplasmic assembly properties, substitution of the head domain of lamin A for that of NF-L did not substantially affect the ability of NF-L to coassemble with vimentin in the cytoplasm. In contrast, insertion of a 42–amino acid sequence unique to lamin rod domains into NF-L profoundly affected NF-L coassembly with vimentin indicating that the 42–amino acid insertion in lamins may be important for sorting lamins from cytoplasmic IF proteins.

INTERMEDIATE filaments (IF)[1] are a heterogenous group of proteins that form 10–nm filaments of the cytoskeleton of eukaryotic cells. Although the elucidation of IF function has been enigmatic, evidence is beginning to accumulate that indicate IF play important roles in cellular processes. For example, the protective function of skin is compromised by single amino acid substitutions in keratin molecules (reviewed by Fuchs, 1994). Other evidence indicates that IF are required for gastrulation, embryogenesis, nuclear organization, DNA replication, nuclear envelope assembly, and the regulation of axonal caliber (Newport et al., 1990; Cleveland et al., 1991; Klymkowsky et al., 1992; Torpey et al., 1992; Ulitzur et al., 1992; Baribault et al., 1993). This diversity of function is presumably determined in part by the heterogenous subunit composition of IF protein assemblies.

A comparison of IF proteins reveals a common tripartite organization consisting of a central moderately conserved α-helical rod domain, flanked by less well conserved "head" and "tail" domains. Mammalian IF have been subdivided

into six classes (reviewed in Fliegner and Liem, 1991; Stewart 1990). Class I and II, represented by the type I (acidic) and type II (basic) keratins, respectively, are expressed in epithelial cells. Class III consists of vimentin, expressed in mesenchymal cells; desmin, expressed in muscle cells; glial fibrillary acid protein, expressed in glial cells; and peripherin, expressed in neurons of the peripheral nervous system. Class IV consists of the major neurofilaments (NF), NF-L, NF-M, and NF-H, and α-internexin, all of which are expressed predominantly in neuronal cells. Class V is composed of the nuclear lamins (A, B, and C) which are expressed ubiquitously. Nestin, expressed in neuroendothelial cells, comprises Class VI and shares some properties with Class IV IF proteins.

The dynamics and mechanism by which IF are assembled in vivo are not fully understood (see Shoeman and Traub, 1993). However, some properties of IF are apparent from ultrastructural observations, in vitro assembly, DNA transfection, and immunofluorescence studies. First, the α-helical rod domain, especially the sequence highly conserved at its COOH proximal end, appears essential for filament formation since IF mutants modified in this region fail to assemble into filaments. In addition, the "head" and "tail" domains are also important for assembly since IF mutants deleted in these regions are often compromised in assembly (Albers and Fuchs, 1989; Gill et al., 1990; Wong and Cleveland, 1990; Chin et al., 1991; Letai et al., 1992; Wilson et al., 1992; Heins et al., 1993). Second, keratin filaments cannot

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1. *Abbreviations used in this paper*: DAPI, 4'6-diamindino-2-phenylindole; IF, intermediate filaments; NF, neurofilament; NLS, nuclear localization signal.

be formed by homopolymerization of either type I or type II subunits, but are instead formed by heteropolymerization of equimolar amounts of type I and type II subunits (O'Guin et al., 1990). Third, most members of Class III can form homopolymeric filaments, whereas Class IV members appear to be obligate heteropolymers (Ching and Liem, 1993; Lee et al., 1993). Furthermore, type III and IV intermediate filament members can coassemble in vivo (Chin and Liem, 1989, 1990; Monteiro and Cleveland, 1989). In contrast, Class III members are unable to form heteropolymers with cytokeratins (Osborn et al., 1980; Quax et al., 1985). Similarly, Class V proteins do not coassemble with any other IF member.

The structural determinants that enable IF proteins to sort to different subcellular compartments are poorly understood. However, two possibilities are apparent. The primary sequence of IF proteins themselves may be sufficient to confer segregation. Alternatively, other proteins may be required to facilitate this polymerization.

In an elegant study to determine how keratin and vimentin proteins are differentially sorted, McCormick et al. (1991) constructed a series of chimeric IF molecules containing different combinations of vimentin and keratin I sequences. These chimeric molecules were analyzed after DNA transfection for their ability to segregate into keratin or vimentin filament networks. It was demonstrated that two subdomains of the rod segment, helix 1B and helix 2B, had the greatest ability to confer sorting, although other rod domain sequences also appeared to influence segregation. In addition, their transfection results suggest that cellular factors unique to certain cell types may also influence assembly of IF in vivo.

It is not known whether similar mechanisms exist for sorting cytoplasmic and nuclear IF arrays. Type V IF subunits, the nuclear lamins, are unique in that they assemble as a meshwork of filaments on the inner side of the nuclear envelope (see Gerace et al., 1984; Aebi et al., 1986; Newport and Forbes, 1987; Gerace and Burke, 1988; Burke, 1990). In contrast, all of the other eukaryotic IF are assembled in the cytoplasm. The sorting of nuclear and cytoplasmic IF in the same cell is highly efficient when one considers that at mitosis both the nuclear lamina array (following nuclear envelope breakdown) and cytoplasmic IF arrays are depolymerized and thus the subunits can apparently freely intermingle (Gerace and Blobel, 1980; Chou et al., 1991). Remarkably, after mitosis, cytoplasmic IF and nuclear lamins are sorted from one another despite their apparent structural relatedness (Fisher et al., 1986; McKeon et al., 1986; Stewart, 1990; Parry and Steinert, 1992).

Although the details of how this sorting is achieved are not fully understood, some sequences unique to lamins appear to be involved in the process. Thus two different sequence elements, a nuclear localization signal (NLS) and a -CAAX motif (where C is cysteine, A indicates an aliphatic amino acid, and X is variable) have been shown to be required for nuclear localization and nuclear membrane association of the polypeptides, respectively (Loewinger and McKeon, 1988; Holtz et al., 1989; Krohne et al., 1989; Nigg, 1989; Kitten and Nigg, 1991; Hennekes and Nigg, 1994). Apart from these elements it is not known what other sequences are required to confer nuclear lamina assembly. A comparison of mammalian IF sequences indicates that lamins contain

other features that distinguish them from their cytoplasmic counterparts (see Nigg 1989). First, the central rod domain, 310 amino acids long in cytoplasmic IF, is 42 amino acids longer in lamins due to an insertion of six complete α-helical heptad repeats in the 1B tract of the rod domain (see Figs. 1 and 2). Interestingly, the rod domains of two invertebrate IF proteins, an IF protein in *Helix aspersa* and squid NF also contain a 42-amino acid insertion in their rod domains (Dodemont et al., 1990; Way et al., 1992). Second, lamins contain four conserved tryptophan residues, and an acidic domain in their carboxy-terminal regions.

In this study we tested which lamin sequences are required for directing localization of an otherwise cytoplasmic IF protein to the nuclear lamina. Our results suggest IF sorting is a complex process governed by multiple sequence elements that contribute both to targeting as well as retention in different subcellular compartments.

Materials and Methods

Construction of NF-L Genes Modified with Lamin Sequences

To express NF-L genes in mouse cells we used plasmid pMSV-NF-L, deleted of 11 amino acids of NF-L carboxyl-terminal sequences and tagged with the 12 amino acids of *myc* sequences (termed pNF, Fig. 1 *A*; see Gill et al., 1990) to generate the modified NF-L molecules described below.

pNF(+NLS). To insert the NLS into NF-L, a 57 base primer 5'GCA-TCGGCCGTCCTCCACCTTGCGCTTCTTCTTGGTGGGGAAGACC-TCCGAGCTC TG3' and a 17-base primer 5' CAGGAAACAGCTATGAC3' were synthesized. The primers contained 18 bases at their 3' ends complementary to the sequence encoding amino acids 420–415 in mouse NF-L, and to sequences in pUC19 located upstream of the MSV promoter, respectively. The primers were used to PCR amplify pNF template, and the ~1.9-kbp product was double digested with *BglII* (codon 242 in NF-L) and *EagI*. Note that in this and subsequent PCR reactions, restriction enzyme sites (an EagI site in this case) were introduced by the PCR primers. The 566-bp BglII-EagI product was substituted for the corresponding fragment (between codons 242 and 420) in pNF. This procedure resulted in insertion of amino acids PTKKKRKVED between codons 420–421 in NF-L (see Fig. 1 *B*).

pNF(+NLS+CAAX). To insert the CAAX motif into pNF(+NLS), a 58-base primer 5'TACCGTCGACTCACATGATGCTGCAGTTGGGGCTC-TGGGTATTCAAGTCCTCTTC AGA3' and the 17-base primer (described above) were used to PCR amplify pNF(+NLS). The ~2.3-kbp PCR product was digested with *KpnI* and the 583-bp fragment generated was substituted for the corresponding KpnI (located at codon 368 in NF-L) and *HincII* (located at the beginning of the myc tag) fragment in pNF(NLS). This resulted in the addition of TQSPNCSIM* codons found at the COOH terminus of lamin A onto the myc sequence found at the end of NF-L(+NLS) (see Fig. 1 *C*).

pNF(+42). To insert the 42-amino acid sequence of lamins into NF-L a 77-base primer 5'CGCGAGCTCCAGGAACTCCGCCCGGACCCGCC-GCTACTCCAGCCGGAGGAAGAACCACAACGCTCGGACGTCCAACG and a 96-base primer 5'AGCCTCGAGGCTCTCCTCAACAGCAAGGAAG-CCGCCCTCAGCACCGCCCTGGGCGAGAAGCGCACCCTGGAGG-CCGAGTATGAGGAAGAAGTGCTG3' were synthesized. The two primers contained 18 bases at their 3' ends that were complementary to the six codons upstream of amino acid 177 in NF-L and the six codons downstream of codon 178 of NF-L, respectively. The 77-base primer and the 17-base primer (described above) were used to PCR amplify pNF DNA. The ~1.3-kbp PCR product was double digested with *HindIII* and *XhoI*. The resulting ~1.3-kbp fragment was ligated with the second PCR product that was generated using the 96-base primer, the 57-base primer (described above) and pNF-L as template. The 0.84-kbp fragment from the second PCR was double digested with *KpnI* and *XhoI*. The two PCR digested fragments, ligated by their common XhoI site, were substituted for the HindIII-KpnI fragment of pNF-L. This procedure resulted in insertion of the 42-amino acid sequence NTKKEADLIAAQARLKDLEALLNSKEAALSTALGEKRTL-EAE between amino acids Asn(177) and Tyr(178) in NF-L (Fig. 1 *D*).

pNF(+La/head). To substitute the head domain of NF-L with that of lamin A/C, a 28-base primer 5'GTCGAGTCGGATCCTCCTTCTCCTGC-AG3' and a 29-base primer 5'GCTCGTATCTAGATCTGCAGGACCTCA-AC3' were synthesized. The 28-base primer, and a 17-base primer complementary to PUC19 sequences, were used to amplify the 5' end of lamin A cloned into pUC19 (Fig. 1 *G*). The ~0.2-kbp PCR product was double digested with *EagI* and *BamHI* and ligated with the second PCR product. The 29-base primer and the 57-base primer (described above) were used to amplify the rod domain of pNF-L. The ~1.0-kbp product was digested with *BglII* and the 0.45-kbp 5' rod portion of NF-L was ligated in the appropriate orientation with the EagI-BamHI lamin A PCR product. The ligated fragment was substituted for the 5' EagI-BglII fragment of NF-L. This procedure resulted in precise replacement of the 93-residue head domain of NF-L with the 34-residue head domain of lamin A/C (Fig. 1 *H*).

The rest of the NF-L constructs, pNF(+42+NLS), pNF(+42+NLS-+CAAX), pNF(+La/head+NLS), pNF(+La/head+NLS+CAAX) pNF-(+La/head+42), pNF(+La/head+42+NLS), pNF(+La/head+42+NLS+CAAX) were made by swapping appropriate regions between the NF-L constructs using standard molecular cloning techniques (Fig. 1, *E, F, I, J,* and *M,* respectively).

To express NF-L genes in human SW13 cells, appropriate portions of the above constructs were subcloned into the pBK-CMV expression vector (Stratagene, La Jolla, CA).

Construction of Lamin Genes Modified with NF-L Sequences

To express lamin genes in mouse cells, the complete lamin A cDNA contained on an EagI-KpnI fragment (Fig 1 *G*) was substituted for the 5' EagI and KpnI fragment of NF-L (Fig. 1 *A*). This placed lamin A in the correct orientation for expression from the MSV promoter.

pLa(+NF/tail). A 32-base primer 5'GACTGCACTCTAGAAGCTTGC-GGTAGGCGTGG3' and a 17-base primer 5'ATGGAGACCCCGTCCCA3' were used to PCR amplify the region between codons 1-379 of lamin A. The ~1.15-kbp PCR product was double digested with *AccI* (located at codon 44 in lamin A) and *XbaI* and then ligated with the tail portion of NF-L generated by a separate PCR reaction. For the second PCR reaction, a 30-base primer 5' GCATCCGATCTAGAAGGCGAAGAGACCAGG3' and a 17-base primer 5'GTTTTCCCAGTCACGAC3' were used to specifically amplify the tail domain of NF-L. The PCR product was double digested with *XbaI* and *EcoRI*, and the resulting fragment was ligated with the first PCR restriction product into the EagI-EcoRI sites of pNF-L. This resulted in the substitution of the lamin A tail domain for that of NF-L but in the process the Glu residue at codon 385 was converted to a Thr residue. We considered that this substitution would not be critical for lamin localization, since this amino acid position is variable in different IF proteins (see Fig. 1 *N*).

To construct pLa(+NF/tail+NLS) and pLa(+NF/tail+NLS+CAAX), the tail portion of pLa(+NF/tail) was replaced with NF-L tail domains containing the NLS and CAAX signals from constructs pNF(+NLS) and pNF(+NLS+CAAX) respectively, by standard molecular cloning techniques (Fig. 1, *O* and *P,* respectively).

All of the PCR fragments incorporated in the NF-L and lamin constructs were sequenced and verified to contain the engineered changes. In fact, during sequencing we did not encounter a single error generated by the PCR procedure. It should be noted however, that we discovered several sequence differences in the coding regions of both NF-L and lamin A from the published sequence but these were also present in the cDNA clones from which our chimeric constructs were derived.

Tissue Culture and DNA Transfection

Mouse L tk− cells, and two human SW13 cell lines that either contained or lacked vimentin, cl.1 and cl.2, respectively, were grown in DME supplemented with 10% fetal bovine serum and transfected with plasmid DNAs as calcium phosphate precipitates (Graham and van der Eb, 1973). Stably transfected mouse L cell lines were isolated essentially as described by Monteiro and Cleveland (1989).

Staining of Cells and Analysis by Immunofluorescence Light and Confocal Microscopy

Cells grown on glass coverslips were extracted for 30 s at 37°C in MSB (MSB: 4M glycerol, 100 mM Pipes-KOH, pH 6.9, and 1 mM EGTA) containing 0.5% (wt/vol) Triton X-100 and fixed by immersion in −20°C chilled methanol for 5 min. The cover slips were rehydrated with PBS, in-

cubated for 1 h with primary antibodies, then washed, and after incubation with secondary antibodies, were counterstained with 1 μg/ml 4'6-diamidino-2-phenylindole (DAPI) and mounted onto glass slides using Aqua Poly/Mount (Polysciences, Inc., Warrington, PA). Fluorescence staining of cells was visualized on a Zeiss Axioplan microscope or the BioRad MRC600 confocal laser imaging system attached to a Nikon microscope. Z-series images through cells were generated by viewing 0.7-μm slices taken at successive 0.7-μm intervals.

Primary antibodies used were: mouse monoclonal anti-myc antibody (the mycl-9E10 hybridoma supernatant; Evans et al., 1985); goat polyclonal anti-vimentin antibody (ICN, Costa Mesa, CA), and rabbit anti-lamin (kind gift of Drs. J. Glass and L. Gerace) (Scripps Res. Inst., La Jolla, CA). Primary antibody binding was detected using appropriate affinity-purified fluorescein- and rhodamine-conjugated antibodies (Organon Teknika Corp., Durham, NC).

Protein Preparation, SDS Gel Electrophoresis, and Immunoblotting

Cell lysates were analyzed on 8.5% SDS-PAGE gels as described by Monteiro and Cleveland (1989). Proteins fractionated on polyacrylamide gels were either stained with Coomassie blue or transferred onto nitrocellulose membranes by electroblotting. The filters were reacted with primary antibodies and the immunoblots processed as described by Xiao and Monteiro (1994).

Quantitation of chimeric lamin/NF-L expression in the stable cell line was as described by Gill et al., 1990. For this determination, a bacterial lysate containing twofold serial dilution of *trpE-myc* tagged protein (Wong and Cleveland, 1990) was electrophoresed with two different concentrations of stable cell lysates on 8.5% polyacrylamide gels. After electrophoresis, the proteins were transferred onto nitrocellulose filters and immunoblotted with the myc antibody.

To prepare insoluble and soluble cell fractions, transiently transfected cells (~1-4 × 10⁶) were scraped off the dish with a rubber policeman and collected by centrifugation. The cells were washed with MSB (see above) and resuspended in MSB containing 0.5% (wt/vol) Triton X-100. After 10 min the soluble proteins were recovered by centrifugation. The pellet fraction was resuspended in 50 mM Tris-HCl (pH 6.8) and 0.5% SDS (wt/vol) to equal the supernatant volume. Equal fractions of the supernatant and pellet fractions were analyzed for the presence of myc-tagged proteins by SDS-PAGE and immunoblotting.
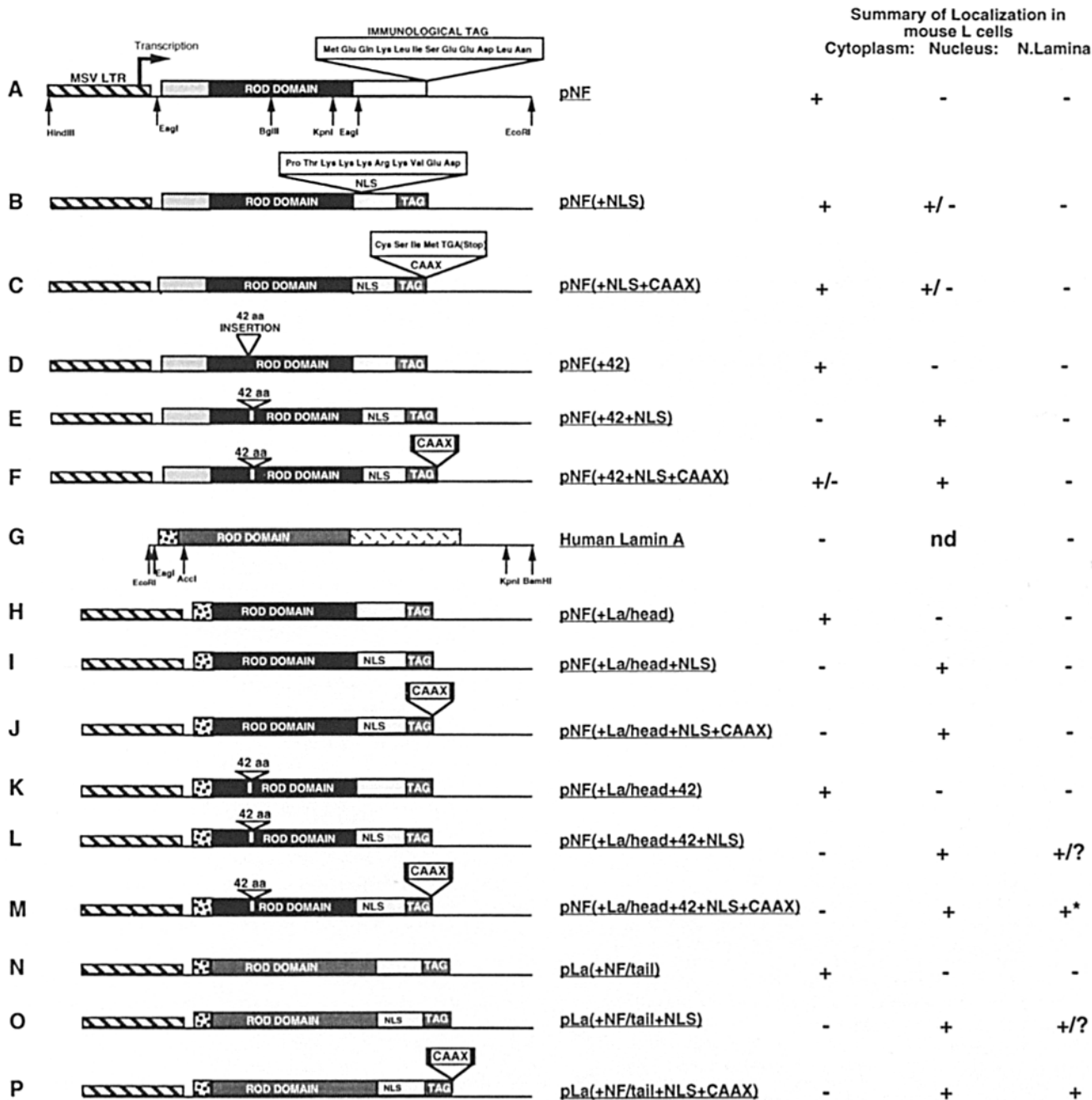
To analyze membrane association of chimeric lamin/NF-L protein in the stable cell line, LA/NF-1, mitotic cells and interphase cells were collected by shake off and scraping, respectively. The cells were washed with cold PBS and the proteins extracted with buffer either containing or lacking Triton X100 (Gerace and Blobel, 1980). For extraction without Triton, cell pellets containing 1-2 × 10⁶ cells were resuspended in 0.25 ml of cold buffer containing 10 mM triethanolamine-HCl (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂ and 0.5 mM PMSF. After 10-min incubation on ice, cells were disrupted in a 0.5 ml homogenizer with 25–30 gentle strokes. 0.25 ml buffer, containing 10 mM triethanolamine-HCl (pH 7.4), 270 mM KCl, 1.5 mM MgCl₂ and 0.5 mM PMSF was added to the homogenate which was subsequently fractionated by centrifugation at 140,000 g in a Beckman TL-100 rotor (Beckman Instr., Carlsbad, CA). For extraction with Triton, cell pellets were resuspended in 0.5 ml of buffer containing 2% Triton X-100, 10 mM triethanolamine-HCl (pH 7.4), 140 mM KCl, 1.5 mM MgCl₂ and 0.5 mM PMSF. The lysate was gently mixed and fractionated as above.

Protein in equal portions of the supernatant and pellet fractions were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with the myc antibody.

Results

### Experimental System for Studying IF Sorting

To determine the structural sequences required for targeting a cytoplasmic IF protein to the nuclear lamina, chimeric molecules of mouse neurofilament (NF-L) cDNAs modified with lamin sequences were constructed (Fig. 1). Mouse NF-L tagged with a myc epitope (Gill et al., 1990) was used as a prototype cytoplasmic IF for this manipulation. Accumulation and distribution of transiently expressed chimeric NF-L proteins was followed by immunofluorescence

Summary of Localization in
mouse L cells
Cytoplasm: Nucleus: N.Lamina

IMMUNOLOGICAL TAG
Met Glu Gln Lys Leu Ile Ser Glu Asp Leu Asn

Transcription
MSV LTR ROD DOMAIN

Pro Thr Lys Lys Lys Arg Lys Val Glu Asp
NLS

Cys Ser Ile Met TGA(Stop)
CAAX

Construct	Name	Cytoplasm	Nucleus	N.Lamina
A	pNF	+	-	-
B	pNF(+NLS)	+	+/-	-
C	pNF(+NLS+CAAX)	+	+/-	-
D	pNF(+42)	+	-	-
E	pNF(+42+NLS)	-	+	-
F	pNF(+42+NLS+CAAX)	+/-	+	-
G	Human Lamin A	-	nd	-
H	pNF(+La/head)	+	-	-
I	pNF(+La/head+NLS)	-	+	-
J	pNF(+La/head+NLS+CAAX)	-	+	-
K	pNF(+La/head+42)	+	-	-
L	pNF(+La/head+42+NLS)	-	+	+/?
M	pNF(+La/head+42+NLS+CAAX)	-	+	+*
N	pLa(+NF/tail)	+	-	-
O	pLa(+NF/tail+NLS)	-	+	+/?
P	pLa(+NF/tail+NLS+CAAX)	-	+	+

Restriction sites labeled: HindIII, EagI, BglII, KpnI, EagI, EcoRI; EcoRI, EagI, AccI, KpnI, BamHI; 42 aa INSERTION; 42 aa

Figure 1. Schematic drawings of NF-L and lamin constructs. *A–F* and *H–M* are mouse NF-L cDNAs tagged with a myc epitope that were modified with lamin sequences. *N–P* are lamin A cDNAs modified with tail sequences derived from constructs *A–C*, respectively. Some restriction sites in constructs *A* and *G* that were used for DNA cloning are shown. Transcription of all the constructs, except construct *G*, were under MSV LTR control [▨]. A summary of the location of the protein product is shown to the right of each construct. +, presence; −, absence; nd, not determined; +/?, lamina-like staining (see text). Note: construct *M* gave lamina staining in only 10% of transfected cells (+*).

and immunoblotting using the monoclonal antibody 9E10, specific for the myc tag. As was found previously, myc-tagged NF-L assembled into cytoplasmic IF arrays in mouse L cells which were counterstained with an anti-vimentin antibody (see Fig. 3 *a*; Gill et al., 1990; M. J. Monteiro, data not shown). This is consistent with coassembly of NF-L with the endogenous vimentin expressed in L cells (Monteiro and Cleveland, 1989; Chin and Liem, 1989; Lee et al., 1993).

In contrast, the control staining for lamins in L cells displayed nuclear staining with rim fluorescence, although a number of apparently intense bright aggregates within the nucleus were also seen (see Fig. 10 *a*). The structural arrangement of IF proteins in these aggregates is not known, but the aggregates have been found in a number of different cells (Goldman et al., 1992; Belmont et al., 1993; Bridger et al., 1993).
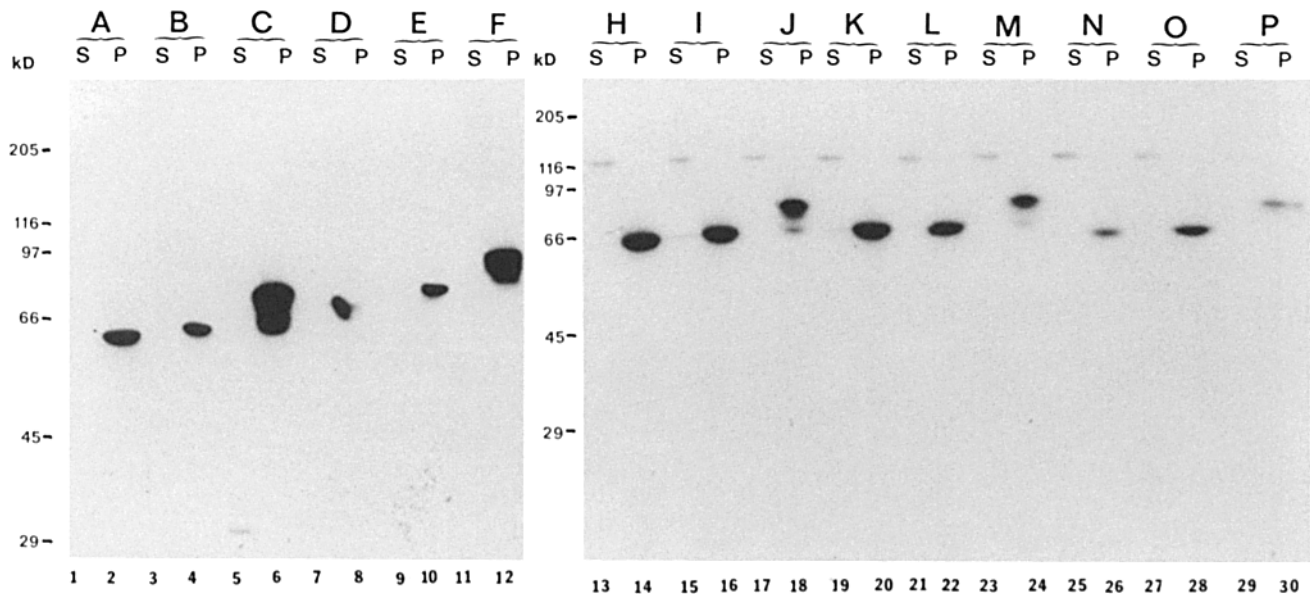
Figure 2. Expression of chimeric NF and lamin polypeptides in mouse L cells. Mouse L cells transfected with the NF-lamin chimeric molecules (listed according to alphabets used in Fig. 1) were harvested and soluble (*S*) and insoluble (*P*, pellet) fractions were separated through 8.5% polyacrylamide gels and immunoblotted with the anti-myc antibody. The position of protein molecular weight markers are indicated, rabbit muscle myosin (205 kD), *Escherichia coli* α-galactosidase (116 kD), rabbit muscle phosphorylase B (97.4 kD), bovine albumin (66 kD), egg albumin (45 kD), and carbonic anhydrase (29 kD). Bar, 20 μm.

NF-L Containing a Nuclear Localization Signal Coassembles with Vimentin in Transfected Cells But Is Not Transported Efficiently into the Nucleus

We next inserted sequences unique to lamins, independently and also in combination with one another, into the tagged NF-L molecule (see Materials and Methods).

We first tested the effect of introducing a nuclear localization signal (NLS) into NF-L. Since the effectiveness of the NLS is influenced by its location within a polypeptide (Roberts et al., 1987), we introduced the signal at position 420 in NF-L, analogous to its position in lamins. Upon transfection into L cells, NF(+NLS) protein was found exclusively in the cytoskeletal fraction and as expected, had a slightly increased molecular mass compared to NF(tag) polypeptide (Fig. 2, lanes 2 and 4). Indirect immunofluorescence microscopy revealed NF(+NLS) was localized in the cytoplasm of transfected cells, although some accumulation was found as multiple aggregates within the nucleus (Fig. 3 *d*). In general, cells analyzed at shorter time points after transfection (10 and 17 h) expressed NF(+NLS) predominantly in the cytoplasm, whereas accumulation within the nucleus was evident in cells either expressing high amounts of the protein, or those analyzed at later time points.

There are two possible reasons for the failure of NF-(+NLS) to be transported efficiently to the nucleus. (*A*) the NLS may not have been recognized by the nuclear transport machinery due to its location or masking in NF(+NLS). (*B*) NF-L may have an extremely high affinity for assembly with the endogenous cytoplasmic vimentin array. In the latter hypothesis the machinery for transport of proteins to the nucleus would be predicted to have a lower affinity (or binding energy) than the assembly (or binding energy) of the protein into the vimentin array.

To test the hypothesis that association with vimentin is

responsible for retention of NF(+NLS) in the cytoplasm, pNF(+NLS) was expressed in two different human cell lines, SW13vim+ and SW13 vim−, that either possess or lack vimentin, respectively (Fig. 4, *a–f*; Sarria et al., 1990). Upon transfection into SW13vim+ cells, NF(+NLS) was found again in the cytoplasm but there was also some accumulation within the nucleus (Fig. 4 *g*). In contrast, when expressed in SW13vim− cells, NF(+NLS) was found exclusively in the nucleus where it appeared to accumulate as multiple spherical aggregates (Fig. 4 *j*). These data indicate that transport of NF(NLS) to the nucleus was governed by its ability to coassemble with vimentin.

We next inserted the CAAX motif, found in most lamins, into pNF(+NLS) to see if this would promote targeting to the nuclear lamina. Immunoblotting of extracts from mouse L cells transfected with pNF(+NLS+CAAX) revealed two polypeptides of 69- and 80-kD reactive with the myc antibody (Fig. 2, lane 6). Reactivity of the bands varied, but in general the higher band predominated. We speculate that the two bands arise due to differences in posttranslational modification. The CAAX sequence is a target for at least three different modifications; the addition of an isoprenyl group to the sulfur atom of the cysteine, cleavage of the last three amino acid residues, and subsequent O-methylation of the carboxy-terminus (see Nigg et al., 1992). At present it is not clear what modification is associated with which of the two bands.

L cells transfected with pNF(+NLS+CAAX) had twisted and distorted filamentous staining in the cytoplasm (Fig 3 *g*). This staining was suggestive of filaments tethered to cytoplasmic membranes, possibly the endoplasmic reticulum. Little, if any, nuclear staining was observed. The membranous staining suggests that the transfected protein was probably isoprenylated since this modification facilitates membrane association (Magee and Hanley, 1988; Nigg et al.,
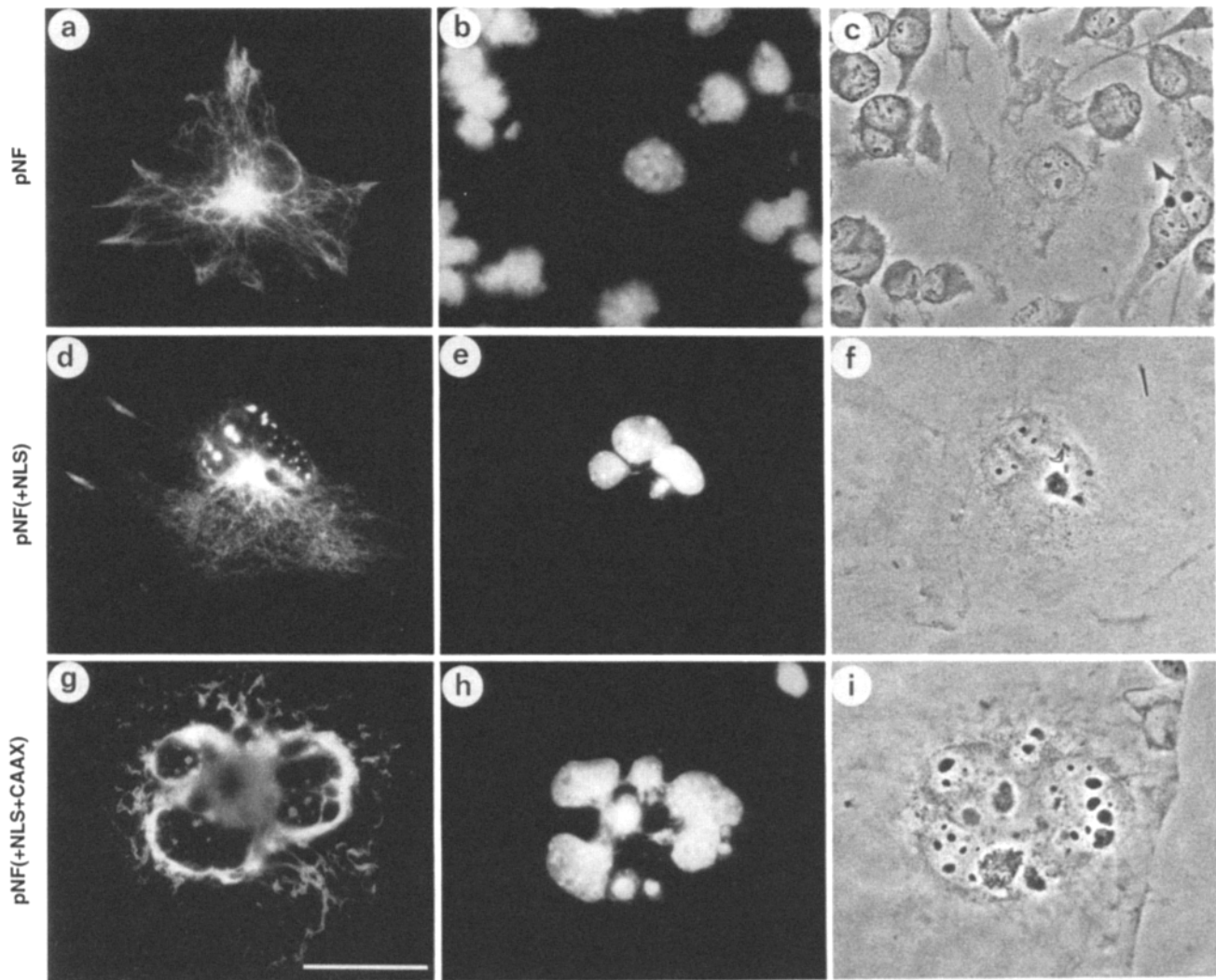
Figure 3. Insertion of a NLS alone is not sufficient to direct nuclear localization of NF-L. Mouse L cells transfected with pNF (*a–c*), pNF(+NLS) (*d–f*), or pNF(+NLS+CAAX) (*g–i*) were stained with the anti-myc antibody followed by fluorescein-conjugated rabbit anti-mouse IgG. The panels show the same cells viewed for fluorescein fluorescence (transfected product; *a, d,* and *g*), DAPI fluorescence (nuclear staining; *b, e,* and *h*) and phase contrast image (*c, f,* and *i*). Bar, 20 μm.

1992; Marshall, 1993; Hennekes and Nigg, 1994). Many of the transfected cells expressing the NF(+NLS+CAAX) protein, including a number of the subsequent constructs to be described, were multinucleated, suggesting that expression of these modified IF proteins caused abnormalities in cell cycle events (e.g., Figs. 3, *e* and *h*, 5 *d*, and 6 *h*). However, when taken together, these data indicate that addition of a NLS and a CAAX motif onto NF-L was not sufficient to direct localization to the nuclear lamina.

Increasing the Length of the Rod Domain of NF-L to that of Nuclear Lamins Causes Diverse Effects on NF-L Assembly and Nuclear Transport

The ability of NF-L to colocalize with vimentin but not with lamins, despite insertion of a NLS or a CAAX motif, may have been governed in part by differences in the lengths of their rod domains. To determine if the length of the rod domain influenced lamina targeting, we inserted into NF-L a 42–amino acid sequence, corresponding to the extra rod sequence present only in mammalian lamins. This 42–amino

acid sequence, containing six heptad repeats predicted to form an α-helix, was inserted in coil 1B of the rod domain of pNF, pNF(+NLS) and pNF(+NLS+CAAX), at precisely the point where NF-L diverges from lamins.

An immunoblot of proteins from mouse L cells transfected with these constructs is shown in Fig. 2 (lanes 7–12). In both pNF(+42) and pNF(+42+NLS) a single protein band, 4.5 kD larger than the parental molecules, was detected in cytoskeletal fractions. In contrast, 2 polypeptide bands were visualized in the insoluble extract from cells transfected with pNF(+42+NLS+CAAX), a feature common to all transfections involving CAAX-containing constructs. All subsequent constructs analyzed encoded polypeptides of expected size and were recovered predominantly in the insoluble fraction (Fig. 2).

In a small proportion of cells (∼3–5%), correlating with low protein expression (inferred by weak staining), NF(+42) appeared to associate and/or assemble into a filamentous cytoplasmic array (Fig. 5 *a*), which was counterstained with vimentin by double immunofluorescence microscopy (Fig. 5
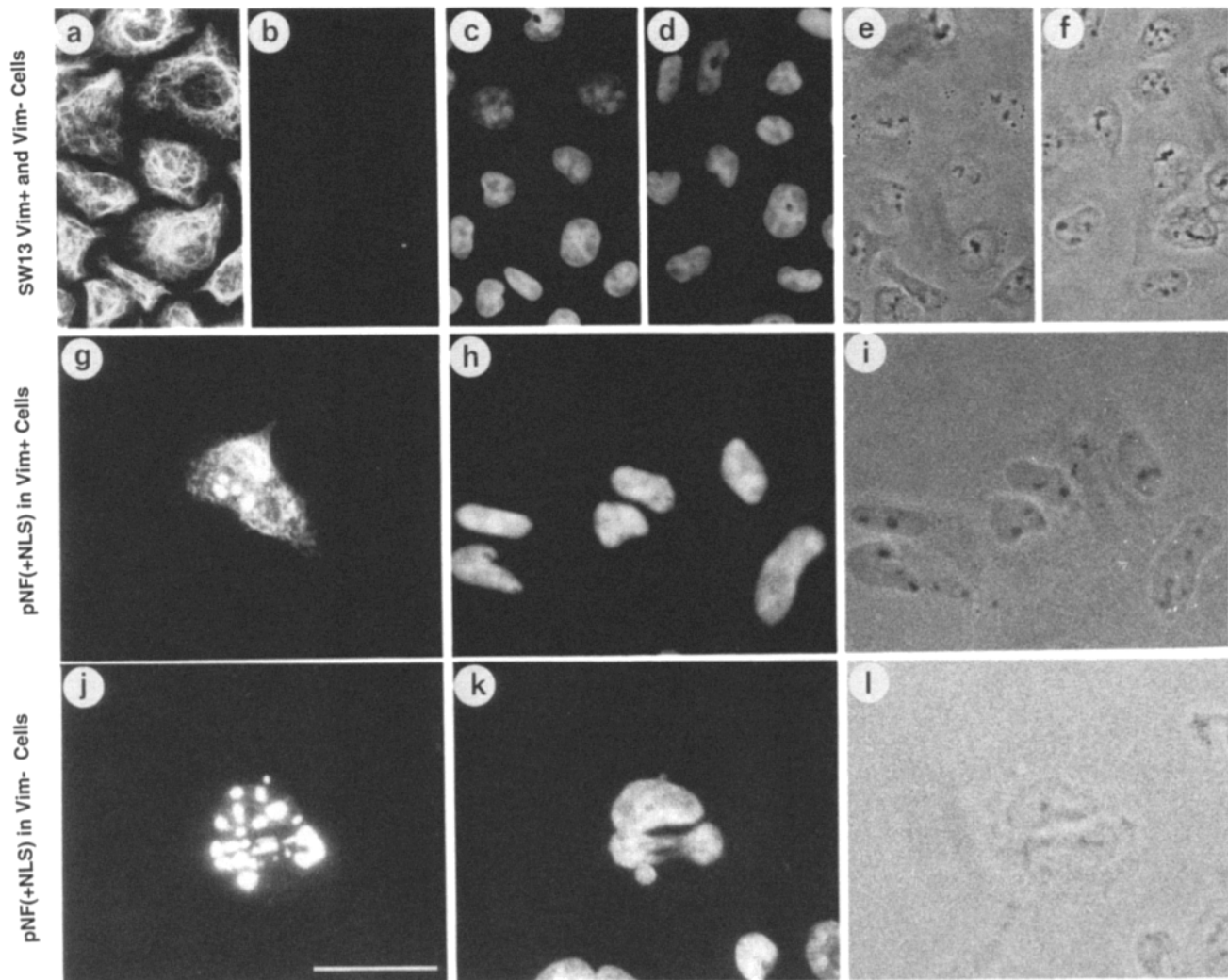
Figure 4. NF-L with a NLS is retained in the cytoplasm due to interaction with vimentin. Two different human SW13 cell lines 1.1 and 1.2 that contain (*a, c,* and *e*) or lack (*b, d,* and *f*) vimentin were transfected with pNF(+NLS). 48 h after transfection the cells were stained for the transfected NF-L product using the myc antibody (*g* and *j*). *a* and *b* are SW13 1.1 and 1.2 cells stained for vimentin. The corresponding DAPI and phase contrast images of these cells are shown in *c-f*, respectively. *g* and *j* (fluorescein), *h* and *k* (DAPI), and *i* and *l* (phase contrast image) are SW13 cells transfected with pNF(+NLS). Bar, 20 µm.

b). This association or assembly was compromised when higher amounts of the mutant protein was expressed (inferred by strong staining; see the cell towards the bottom in Fig. 5 *a*).

Further insertion of a NLS onto pNF(+42) altered the distribution of the protein to an exclusively nuclear location (Fig. 5 *c*). This change was in sharp contrast to NF(+NLS) which had a predominantly cytoplasmic localization in mouse L cells (Fig. 3 *d*). Interestingly, although pNF(+42+NLS) was transported into the nucleus, it did not appear to colocalize with nuclear lamins even though their rod lengths were equivalent. Instead, pNF(+42+NLS) was either distributed uniformly across the nucleus or accumulated into a number of crescent shaped structures.

Localization to the nuclear lamina was not facilitated even when a CAAX motif was added onto NF(+42+NLS). Unlike the lamina staining shown in Fig. 10 *a*, cells transfected with pNF(+42+NLS+CAAX) had punctate staining juxtaposed to nuclear membranes (Fig. 5 *f*). In some cells where

expression was increased, additional staining was found in the cytoplasm, but this too appeared to be associated, at least in part, with nuclear membranes. These data suggest that the 42–amino acid sequence facilitated nuclear targeting, but was insufficient for lamina localization of NF-L.

The Role of the Head Domain of Nuclear Lamins in IF Assembly

A number of studies imply that the head domain of IF proteins may be more important for IF assembly than the tail domain (Traub and Vorgias, 1983; Kaufmann et al., 1985; Gill et al., 1990; Wong and Cleveland, 1990; Traub et al., 1992; Shoeman and Traub, 1993; however see McCormick et al., 1993). We therefore investigated whether lamina targeting would be facilitated by inclusion of the head domain of lamin A onto NF-L.

Six different constructs containing this substitution were analyzed for lamina targeting. The first three contained the
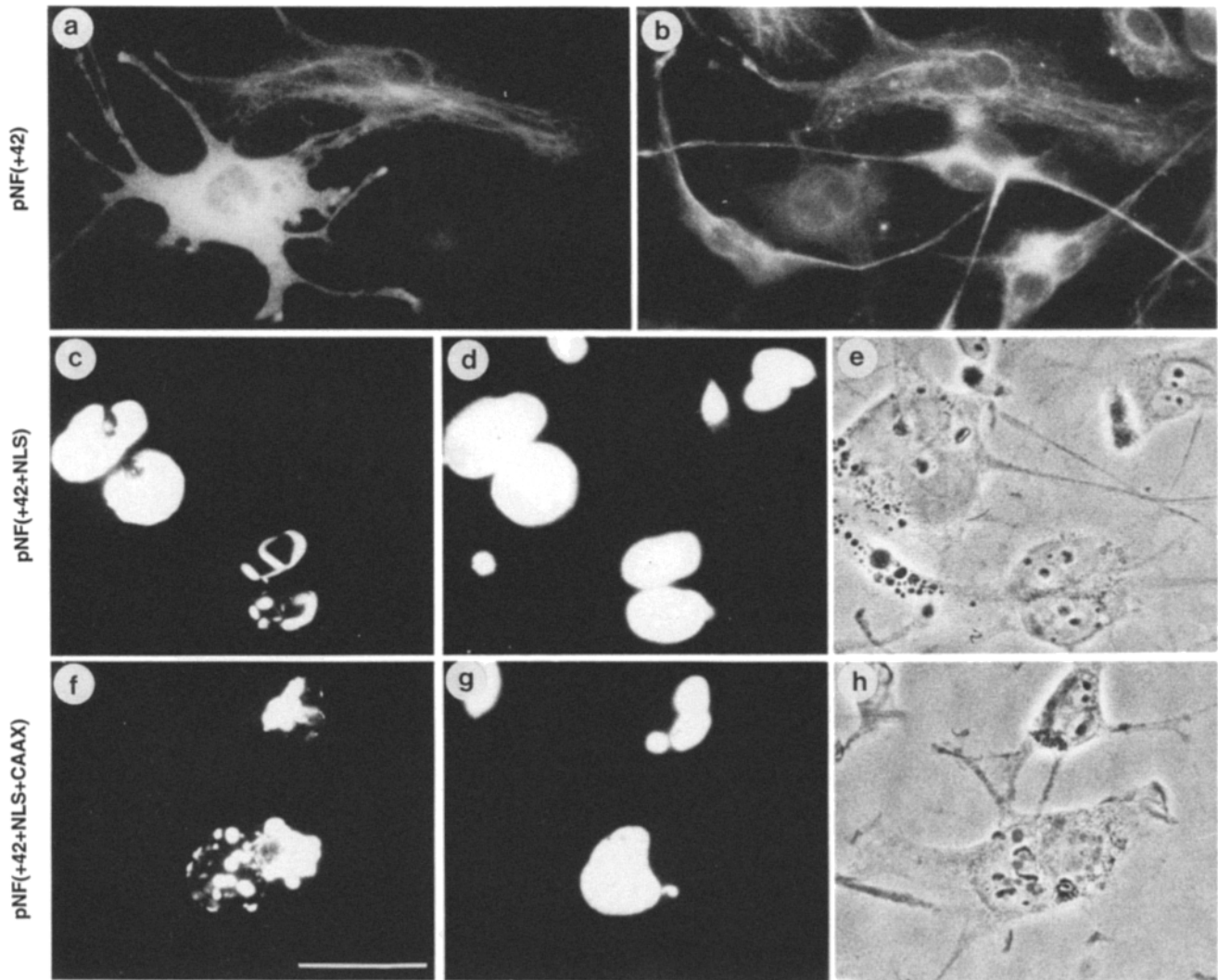
Figure 5. Insertion of a 42–amino acid sequence into the rod domain of NF-L weakens the interaction of NF-L with vimentin. Mouse L cells were transfected with pNF(+42) (*a* and *b*), pNF(+42+NLS) (*c-e*), or pNF(+42+NLS+CAAX) (*f-h*). Anti-myc fluorescence (fluorescein staining; *a*, *c*, and *f*); vimentin fluorescence (rhodamine staining; *b*), DAPI fluorescence (*d* and *g*); and phase contrast images (*e* and *f*). Bar, 20 μm.

wild-type rod domain of NF-L, but differed in that the first contained the tail domain of NF-L (tagged with myc sequences), the second contained the tail of NF-L plus the NLS, and the third contained the tail of NF-L plus both the NLS and the CAAX motif (Fig. 1, *H-J*). Three additional constructs were made that contained the 42–amino acid insertion into the rod domain of NF-L, but were otherwise identical to the first three constructs (Fig. 1, *K-M*).

Upon transfection in L cells, pNF(+La/head) was efficiently assembled into filaments (Fig. 6 *a*). Double immunofluorescence staining indicated that the chimeric NF(+La/head) molecules colocalized with vimentin (data not shown). However, substitution of the head domain weakened the interaction of NF-L with vimentin since further addition of a NLS signal resulted in transport of more NF-L into the nucleus than was found with pNF(+NLS) (compare Fig. 6 *d* with Fig. 3 *d*). When the CAAX motif was added onto the pNF(+La/head+NLS) the protein accumulated around nuclei, but careful examination of the phase image indicated

that most, if not all, the staining was on the cytoplasmic side of nuclei (Fig. 6, *g-i*). Interestingly, many of the cells transfected with this construct were found to have abnormal nuclei (Fig. 6 *i*).

The constructs containing the 42–amino acid insertion were next examined for lamina targeting. Chimeric NF(+La/head+42) containing the 42–amino acid insertion did not assemble into any discernible IF array and displayed diffuse cytoplasmic staining (Fig. 7 *a*). Despite this apparent lack of filament assembly, the protein was recovered in the insoluble cytoskeletal fraction (Fig. 2, lanes *19* and *20*). Upon addition of a NLS signal, the 42–amino acid insertion mutant accumulated within nuclei, where it formed a reticular meshwork (Fig. 7 *d*). These nuclei lacked the bright rim fluorescence staining typically seen with lamin A/C antibody staining (compare Figs. 7 *d* with 10 *a*). Consequently confocal microscopy was used to determine whether NF(+La/head +42+NLS) was present throughout the nucleoplasm or restricted to certain regions of the nucleus. A series of 0.7-
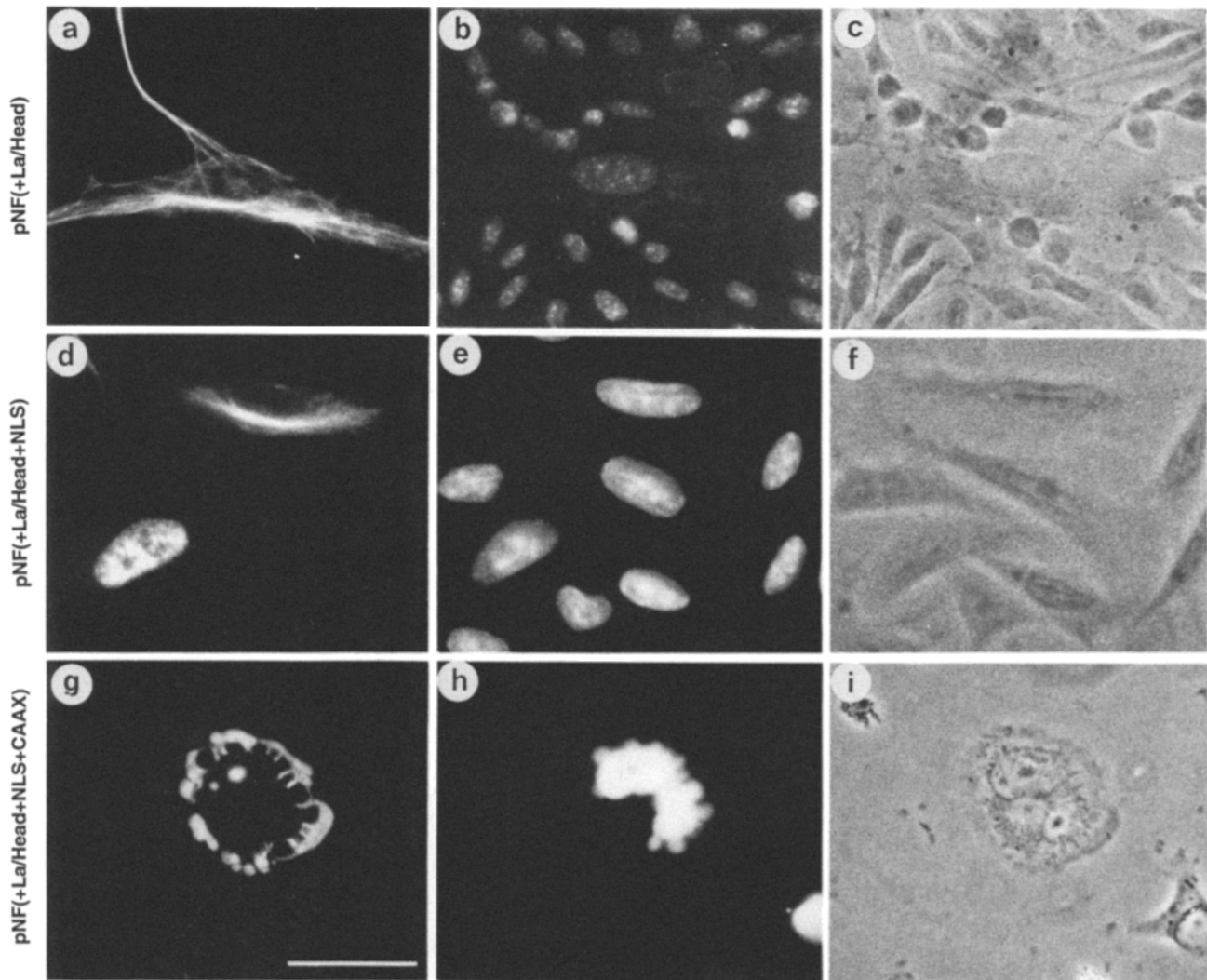
Figure 6. Substitution of NF-L head domain with that of lamins does not compromise NF coassembly with vimentin. Mouse L cells were transfected with pNF(+La/head) (*a–c*), pNF(+La/Head+NLS) (*d–f*), or pNF(+La/Head+NLS+CAAX) (*g–i*). Fluorescein staining for the myc transfected products is shown on the left panels together with DNA staining (*center*) and phase contrast images (*right*) of the same set of cells. Bar, 20 μm.

μm optical sections taken through the nucleus revealed NF-(+La/head+42+NLS) was localized with a cage-like distribution beneath the nuclear envelope (Fig. 8, *a–c*).

Further addition of a CAAX motif resulted in ∼10% of cells transfected with pNF(La/head+42+NLS+CAAX) having a staining pattern remarkably similar to that of lamins (Fig. 7 *g*). These nuclei had a rim fluorescence when viewed by light microscopy and confocal microscopy (Fig. 8, *g* and *h*). A larger proportion of cells transfected with pNF(La/head+42+NLS+CAAX) had diverse phenotypes (data not shown). Approximately 80% of transfected cells examined 48 h after transfection had rim staining together with either diffuse cytoplasmic staining or intense staining associated with the nuclear periphery. In the remaining cells (∼10–15%) the chimeric NF(La/head+42+NLS+CAAX) protein was located in large spherical structures within nuclei. In some cases the spherical structures also displayed rim staining (data not shown). However, the rim fluores-

cence of the inclusions did not coincide with the nuclear envelope by phase microscopy (data not shown).

The Tail Domain of Lamin A Is Not Essential for Nuclear Lamina Localization

In order to determine if the tail domain of lamins was required for lamina targeting, we used the complementary approach of fusing the body of lamin A with different combinations of tail sequences derived from NF-L.

To construct chimeric lamin–NF-L molecules, the complete human lamin A cDNA (Fig. 1 *G*) was first placed under the control of the MSV promoter. The entire tail domain of lamin A was then precisely substituted with the complete tail domain of NF-L (containing the hc-myc tag) resulting in construct pLa(+NF/tail) (Fig. 1 *N*). Two additional constructs were made. pLa(+NF/tail+NLS) (Fig. 1 *O*) contained a NLS within the tail domain of NF-L, and pLa-

Monteiro et al. *Sorting of Nuclear and Cytoplasmic Intermediate Filaments*   1335
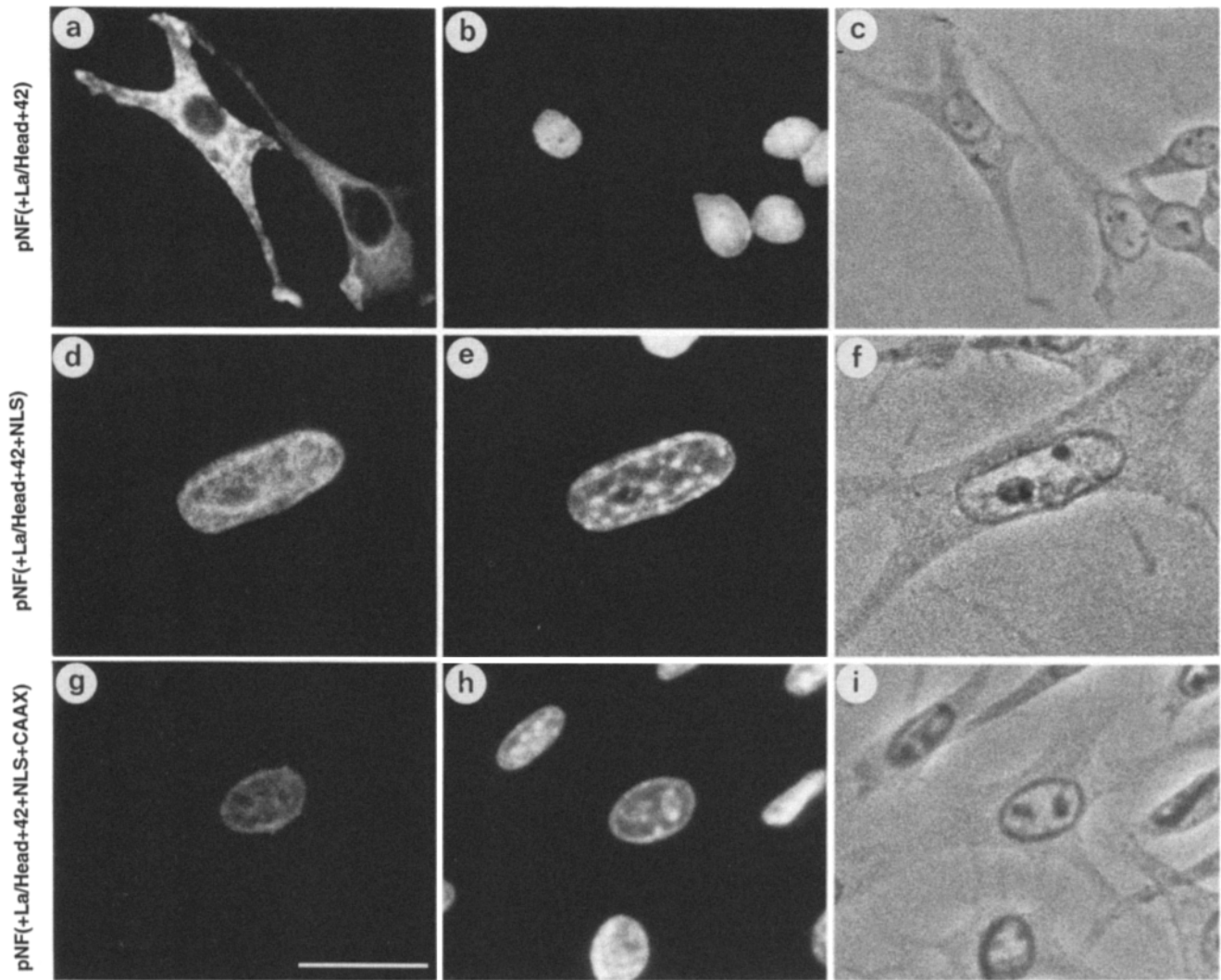
Figure 7. NF-L containing a substituted lamin head domain and the 42–amino acid lamin extension in helix 1B, together with an inserted NLS and CAAX motif, apparently localizes correctly to the nuclear lamina in a limited number of cells. Mouse L cells were transfected with pNF(+La/head+42) (*a–c*), pNF(+La/Head+42+NLS) (*d–f*), or pNF(+La/Head+42+NLS+CAAX) (*g–i*). Fluorescein staining for the myc transfected products are shown on the left panels together with DNA staining (*center*) and phase contrast images (*right*) of the same set of cells. Bar, 20 μm.

(+NF/tail+NLS+CAAX) (Fig. 1 *P*) contained both the NLS and the CAAX motif. The localization of each chimeric protein was analyzed following transfection into mouse L cells.

Chimeric lamin A containing a substituted NF-L tail domain, pLa(+NF/tail), failed to localize to the nucleus but instead formed giant filamentous structures in the cytoplasm (Fig. 9 *a*). This change in localization is consistent with deletion of the NLS contained in the tail domain of lamin A (Loewinger and McKeon, 1988). The results further demonstrate that the NF-L tail sequence used in the construction of the chimeric protein was devoid of a NLS.

When a NLS was inserted into the substituted tail of NF-L, the chimeric lamin A protein, LA(+NF/tail+NLS), was transported into the nucleus (Fig. 9 *d*) where it appeared to form a meshwork of filaments that were localized as a cage

just beneath the nuclear envelope (Fig. 8, *d* and *e*) similar to that previously seen for pNF(La/head+42+NLS). Therefore, apart from the lack of a rim fluorescence by light microscopy, the staining appeared for the most part to be similar to that of lamins.

Additional evidence suggesting that most of the lamin A tail domain is dispensable for lamina targeting was obtained upon transfection of pLa(NF/tail+NLS+CAAX). This chimeric protein was found exclusively within nuclei of transfected cells (Fig. 9 *g*). The nuclei had a prominent rim fluorescence by light and confocal microscopy which was indistinguishable from that of wild type lamin staining (Fig 10 *a*). Most of the cells examined 48 h after transfection had this staining pattern with normal looking nuclei, however, a small proportion of transfected cells (∼5%) had grossly distorted, and sometimes fragmented, nuclei (data not shown).
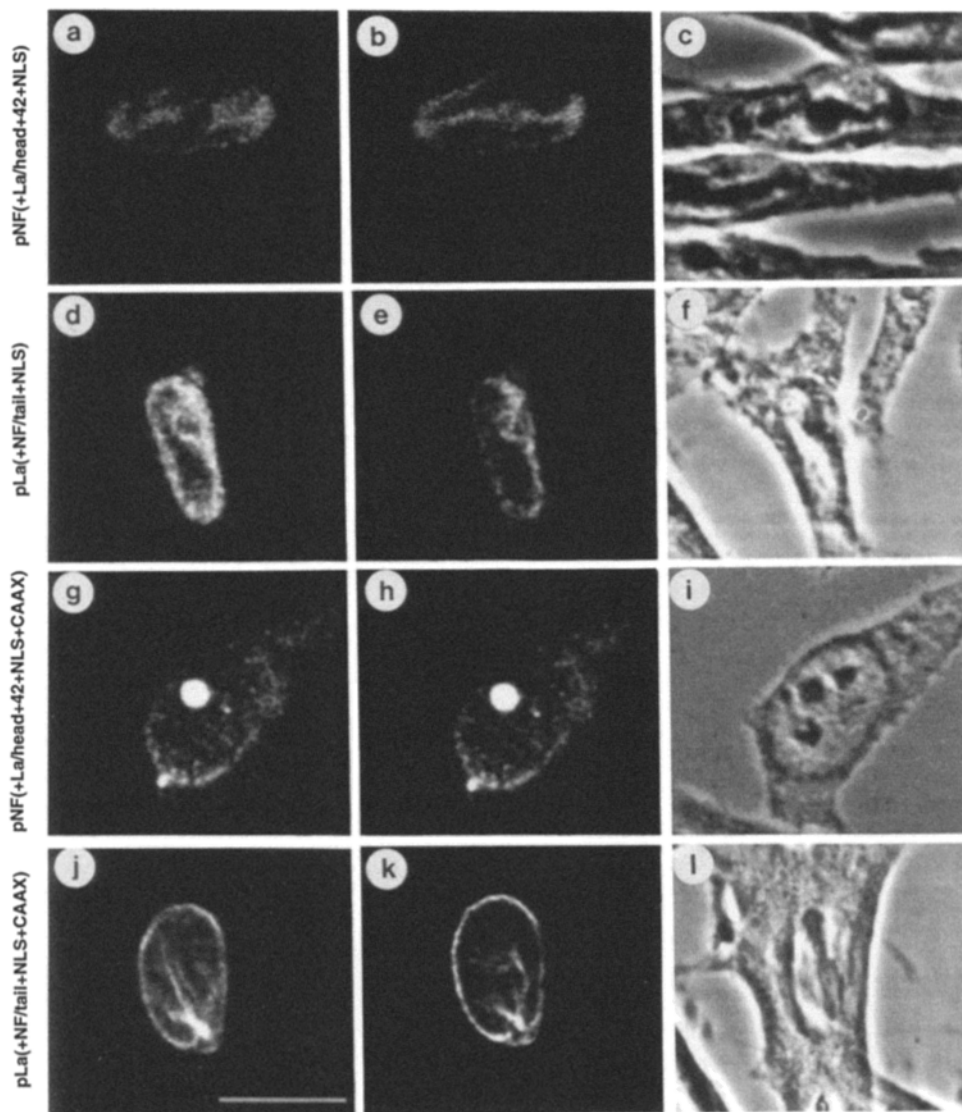
Figure 8. Confocal microscopy of transfected cells shows lamina like staining of modified NF and lamin molecules. Mouse L cells transfected with pNF(+La/Head +42+NLS) (*a*, *b*, and *c*), pLA(+NF/tail+NLS) (*d*, *e*, and *f*), pNF(+La/Head+42 +NLS+CAAX) (*g*, *h*, and *i*), or pLA(+NF/tail+NLS +CAAX) (*j*, *k*, and *l*) were viewed by confocal laser imaging for the myc tagged polypeptides. Left and center panels fluorescein fluorescence of successive 0.7-μm sections taken through the nucleus of individual transfected cells. Right panels are the corresponding phase contrast images. Note the cage-like distribution of the transfected products. In some cells, apart from a rim staining, intranuclear fluorescence was also seen which correlated with infolds of the nuclear membrane. Bar, 10 μm.

Many of the latter cells had increased staining, suggesting that high levels of the chimeric protein may have caused this phenotype.

Isolation of a Stable Cell Line Expressing Chimeric La(+NF/Tail+NLS+CAAX): Conversion from a Lamin A to B Type Lamin

Although chimeric La(+NF/tail+NLS+CAAX) protein appeared by microscopy to be targeted appropriately to the nuclear lamina, it was not clear whether expression would adversely affect cells in terms of their viability or cell cycle events. In order to address such questions mouse L-cells were cotransfected with the pLa(+NF/tail+NLS+CAAX) and pSVneo DNAs, and G418 resistant cell lines were isolated. One of four cell lines analyzed by immunoblotting was found to express the chimeric protein. This cell line had growth rates and morphology similar to that of the parent cell line. The amount of chimeric protein expressed by this stable cell line, LA/NF-1, was determined by quantitative

immunoblotting to constitute ∼1.8% of total cell protein (Fig. 10 *B*).

As expected, immunofluorescence microscopy revealed that all LA/NF-1 cells were positive for the chimeric protein (data not shown). In interphase cells, the protein was found only in nuclei and had a staining pattern indistinguishable from that of wild-type lamins. However, in mitotic cells the chimeric protein did not behave like its parent, lamin A, but instead behaved like lamin B. During nuclear envelope disassembly in mitosis, lamin B remains associated with membrane remnants of the nuclear envelope, whereas lamin A and C both exist in solution as protomers (Gerace and Blobel, 1980). Fig. 10, *c* and *d*, shows a LA/NF-1 cell in late telophase where the chimeric protein is found associated with particulate material. The staining is reminiscent of the behavior of lamin B (see Foisner and Gerace, 1993) suggesting association of the chimeric protein with membrane like particles.

The association of the chimeric protein with membranes was investigated using the biochemical criteria developed by
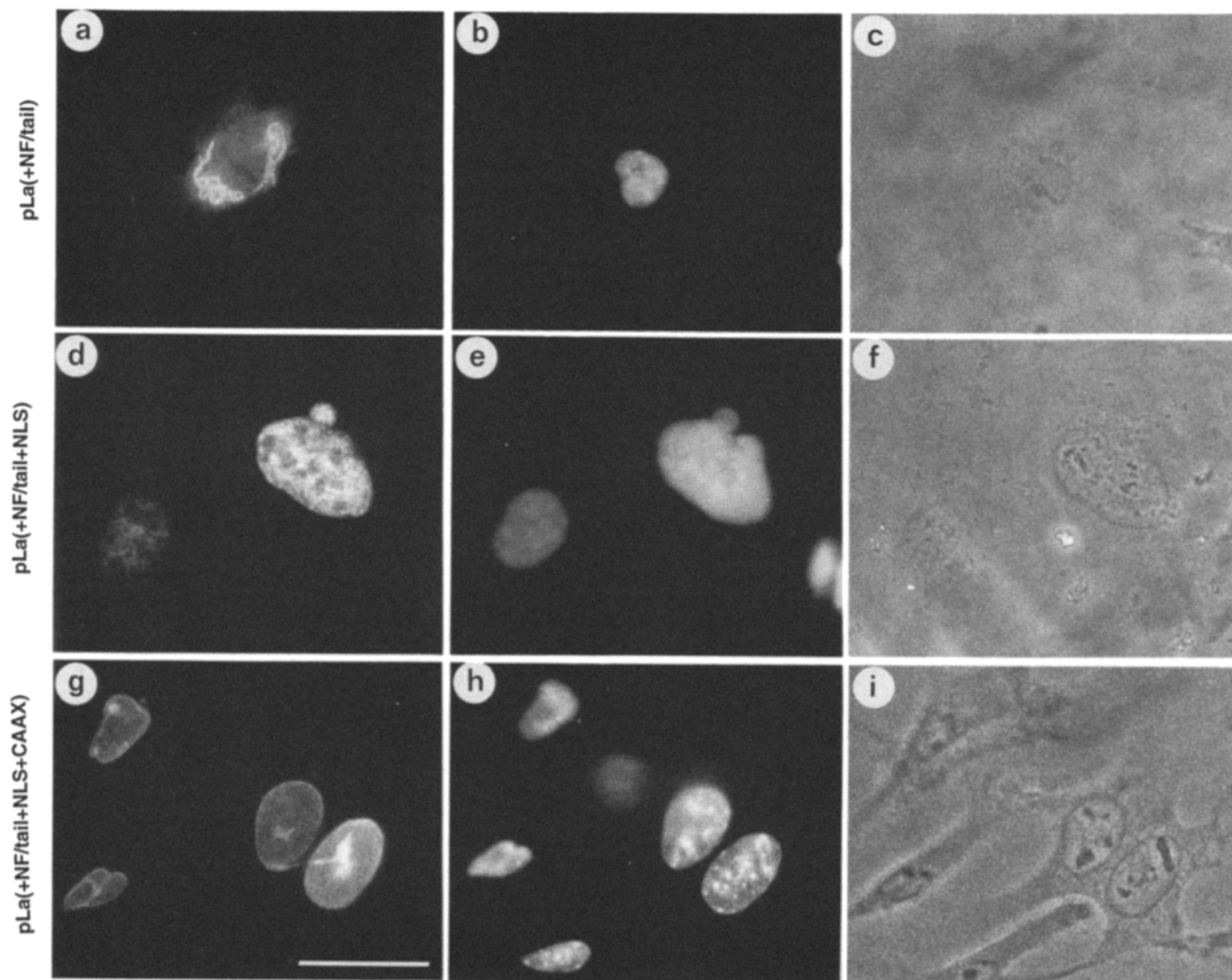
Figure 9. The lamin tail domain is largely dispensable and can be functionally substituted by tail sequences of NF-L. Mouse L cells were transfected with pLa(+NF/tail) *(a–c)*, pLa(+NF/tail+NLS) *(d–f)*, or pLa(+NF/tail+NLS+CAAX) *(g–i)*. Fluorescein staining for the myc transfected products are shown on the left panels together with DNA staining *(center)* and phase contrast images *(right)*. Bar, 15 μm.

Gerace and Blobel (1980). Thus, interphase and mitotic LA/NF-1 cells were lysed separately in buffers containing or lacking Triton X-100 and supernatant and pellet fractions were obtained after high speed centrifugation. Under these conditions, Gerace and Blobel (1980) showed that lamin B in mitotic cells remained attached to membranes that pelleted in the absence of Triton, but was extracted into solution when treated with Triton. Under similar conditions they showed that lamins A and C were soluble in mitotic cells, even in the absence of detergent treatment. Equivalent portions of the soluble and insoluble fractions from interphase and mitotic LA/NF-1 cells were analyzed by immunoblotting to determine the mode of fractionation of the chimeric protein. In the absence of detergent, the chimeric protein was found in the insoluble fraction of both interphase and mitotic cells (Fig. 10 *C*, lanes *2* and *6*). Conversely, when Triton was added during lysis of mitotic cells, almost all of the chimeric protein was extracted into the soluble fraction (Fig. 10 *C*, lanes *7* and *8*). Furthermore, in interphase cells the chimeric protein was found in the pellet fraction independent of de-

tergent treatment (Fig. 10 *C*, lanes *3* and *4*). These results, taken together with the immunofluorescence staining, strongly suggest that pLa(+NF/tail+NLS+CAAX) behaves like lamin B.

Discussion

We have investigated how nuclear and cytoplasmic intermediate filament proteins are sorted in vivo. A simple comparison of sequences between cytoplasmic and nuclear IF proteins reveals regions of identity as well as regions of dissimilarity between the two groups. The most striking differences are in the lengths of the rod domains and the presence or absence of a NLS, a CAAX motif, and four conserved tryptophan residues in the COOH-terminal regions. Sequence differences are also found in the rod as well as head and tail domains. However, these latter differences were in regions of the molecules that show considerable variation between individual members of each group. From this comparison it is evident that any one difference might contribute to

A

B

C
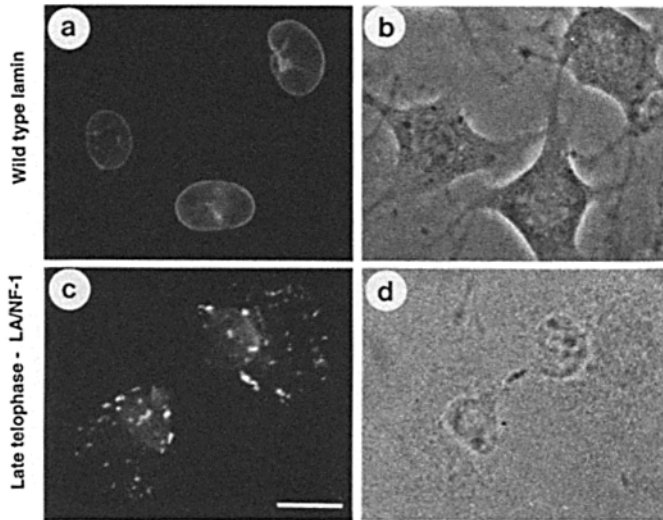
Figure 10. Isolation of a cell line stably expressing La-(+NF/tail+NLS+CAAX): demonstration that the modified lamin A molecule has lamin B-like properties. (*A*) *a* (fluorescein staining) and *b* (phase image) are mouse L cells stained with a rabbit antibody specific for lamins; *c* (fluorescein staining) and *d* (phase image) are of the stable cell line LA/NF-1 in late telophase stained with the mouse monoclonal anti-myc antibody. Please note the presence of a mid-body in *d*. (*B* and *C*) Quantitation and biochemical fractionation of chimeric protein La(+NF/tail+NLS+CAAX) in the stable cell line LA/NF-1. (*B*) Stable cell line lysates were electrophoresed through a 8.5% polyacrylamide gel and immunoblotted along with bacterial lysates containing *trpE-myc* fusion protein. Lanes *1* and *2*, bacterial lysates containing 375 and 150 ng of *trpE-myc* fusion protein, respectively. Lanes *3* and *4*, stable cell line lysates containing 8.5 and 17 µg of total protein, respectively. (*C*) Lanes *1-4*, extraction of interphase LA/NF-1 cell extracts with or without Triton X-100. *S* and *P*, supernatant and pellet resulting from centrifugation of the cell lysate at 140,000 *g*. Lanes *5-8*, extraction of mitotic cells with or without Triton. *B* and *C* were immunoblotted with the anti-myc antibody. Bar, 8 µm.
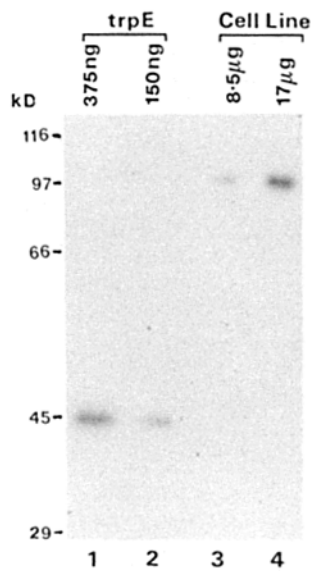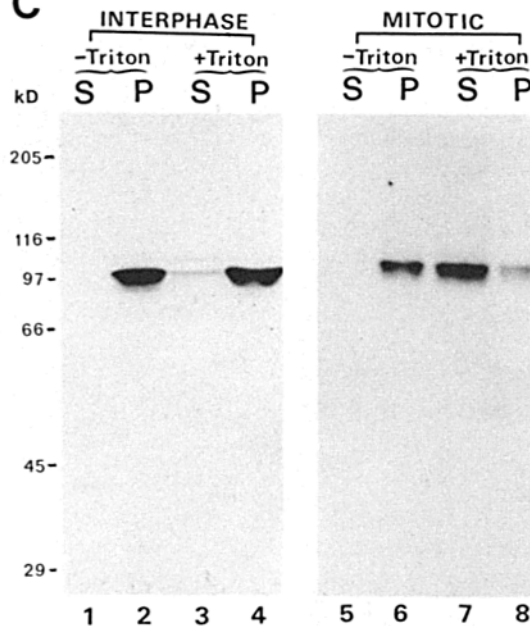
the mechanism by which the two are sorted. Moreover, proteins that interact with the two groups could also influence sorting.

One mechanism we considered crucial for segregation of lamins from cytoplasmic IF proteins was transport of the protein into the nucleus by the NLS. However, the mere insertion of a NLS signal into NF-L was not sufficient to confer transport to the nucleus. Instead, retention of NF(+NLS) in the cytoplasm was governed by expression of another IF protein, vimentin. The mechanism by which vimentin retained NF(+NLS) is not clear. However, the ability of NF-L to coassemble with vimentin suggests a possible mechanism; that is, that NF(+NLS) has a very high rate of coassembly with vimentin, and that this rate must be faster than the time required for transport of the NF(+NLS) into the nucleus. In support of this theory, a number of previous studies have indicated assembly of IF proteins to be rapid (Ngai et al.,

1990; Miller et al., 1991, 1993; Goldman et al., 1992). Alternatively, it is possible that when coassembled with vimentin, the NLS in NF(+NLS) was masked, or the filaments were too large to be efficiently transported into the nucleus. Interestingly, localization of other IF proteins, keratin and plectin, to either the nucleus or the cytoplasm has been shown to be influenced by sequences in the head and tail domains of the molecules (Bader et al., 1991; Wiche et al., 1993). Although it is not known how the domains influenced sorting, it was speculated that interaction of the end domains, especially the COOH domains, with cytoplasmic proteins help keep the molecules from localizing to the nucleus. Thus, the mechanism we propose here for retention of NF-L in the cytoplasm may be common to other proteins, including IF proteins.

When NF(+NLS) entered the nucleus it accumulated as multiple spherical aggregates. This staining differed from

the nuclear rim staining pattern expected for lamins. These results suggest that although these IF proteins are structurally related, NF-L, with a 310-rod domain, cannot colocalize with lamins containing a 352-rod domain. While unlikely, we cannot exclude the possibility of limited co-assembly with lamins in these aggregates. For example, Goldman et al. (1992), showed that lamin A microinjected into cells initially accumulates as foci at the nucleus, which are subsequently incorporated into the nuclear lamina over a period of time. Instead, the aggregates in our experiment probably contained NF-L that could not even self-assemble into filaments. This would be in accord with the recent demonstration that NF-L assembly is dependent on hetero-polymerization with other IF proteins in fibroblast cells (Ching and Liem, 1993; Lee et al., 1993).

The ability of NF-L to coassemble with vimentin was dramatically altered when the 42–amino acid sequence unique to lamins was inserted in the corresponding position in coil 1B of the rod domain of NF-L, thereby extending its rod domain to 352 residues. Reduced affinity for vimentin was evident when a NLS signal was added to this insertion mutant. In contrast to NF-L containing a wild-type rod sequence, the insertion mutant, with the longer rod domain, was efficiently transported into the nucleus even in the presence of vimentin expression. The mechanism for this facilitation is not clear but we suggest two possibilities. First, the insertion may have compromised the ability of NF-L to assemble into filaments. Alternatively, the insertion may have disrupted the higher order assembly of NF-L with vimentin. Evidence both for and against these possibilities was obtained. For instance, when low amounts of the mutant protein lacking a NLS was expressed, it was still able to colocalize with vimentin, at least at the resolution afforded by the light microscope. However, assembly of both the mutant protein and the endogenous vimentin array was compromised with high expression of the insertion mutant. These results reveal an elegant method by which lamins may segregate from cytoplasmic IF proteins. The 42–amino acid sequence unique to lamins contains six putative heptad repeats that are predicted to form an α-helix. Since this insertion failed to strengthen the interaction of NF-L with vimentin, it suggests that it plays an important role in preventing interaction of lamins with cytoplasmic IF arrays, thus ensuring appropriate targeting of lamins to the nucleus. Perhaps the inserted sequence despite being in register with the other α-helical heptad sequences in the rod domain of NF-L, may nevertheless have disrupted coiled-coil interactions with vimentin due to inappropriate charge interactions of the non-hydrophobic residues of the heptad repeats. Indeed structural models of how two α-helices interact indicate that the hydrophobic residues as well as non-hydrophobic residues are important for coiled-coil interactions (McLachlan and Stewart, 1975). Alternatively, it is possible that the head or tail domains are no longer able to interact with their appropriate sequences. Interestingly, models of IF structure suggest that the head or tail domains interact with coil 1B when dimers assemble in a staggered fashion to form filaments (see Heins et al., 1993; Shoeman and Traub, 1993; Steinert et al., 1993; Heins and Aebi, 1994). The addition of the 42–amino acid sequence in coil 1B would clearly displace these normal interactions and thereby affect filament assembly. Paradoxically, we found

that subunits containing the 42–amino acid insertion appeared to colocalize with vimentin when the former was expressed at low levels. This suggests that the two subunits containing a 310 and 352 rod domains can interact, or that these subunits can exchange or are incorporated into filaments, at least in a limited way. The structural details by which this interaction or assembly occurs are at present unclear.

The most appropriate lamina targeting of the modified NF-L molecules we observed was with pNF(+La/Head+42 +NLS+CAAX). However, in this case only 10% of cells had typical lamin staining. More frequently, we observed rim staining together with either diffuse cytoplasmic staining or with intense cytoplasmic staining that was usually associated with the nuclear periphery. In addition, 15% of cells transfected with NF-L altered by the four lamin sequences had large spherical aggregates within the nucleus that resembled, in part, the staining pattern characteristic of lamins. These spherical structures had uniform fluorescence surrounded by a bright rim. This phenotype was previously observed by Loewinger and McKeon, (1988) by a lamin A mutant altered in its rod domain sequence. Both the low levels of proper lamina localization, as well as the abnormal intranuclear structures, suggest that additional lamin sequences are probably required for lamina targeting. The additional sequences that we consider to be crucial for lamina targeting are those located in the rod domain of lamins. The sequences in the tail domain are less likely to be important since we found they were dispensable.

By using the complementary approach of modifying lamin with NF sequences, we showed that the tail domain of lamin A was dispensable for lamina localization. Proper lamina targeting was achieved, provided the substituted tail of NF-L contained a NLS and a CAAX motif. Moreover, the isolation of a stable cell line that expressed this altered lamin as ∼1.8% of total protein suggested nuclear and cell cycle properties were not altered by this change. Since dominant-negative effects on IF assembly have been shown to be induced by expression of as little as half this amount of a mutant IF protein (∼1%; see Wong and Cleveland, 1990), this raises the question of what role, if any, the tail domain of lamins have in cellular processes including assembly or disassembly of the nuclear envelope. Previous studies established that both the head and tail domains contain sites phosphorylated at mitosis and which are crucial for either assembly or disassembly of lamins (Heald and McKeon, 1990; Peter et al., 1990a; Ward and Kirschner, 1990; Luscher et al., 1991). Interestingly, mutation of the phosphorylation site in the tail domain did not appear as dominant as mutation of the head domain site for lamin assembly/dissembly in vivo (Heald and McKeon, 1990). Examination of the substituted tail domain of NF-L indicates multiple Ser/Thr residues that could potentially serve as substitute phosphorylation sites in the chimeric pLa(+NF/tail+NLS+CAAX) protein. However, unlike the lamin phosphorylation sites the putative NF-L tail sites lack the consequence (T/SPXK/R) phospho-acceptor sequence for mitotic cdc2 kinase (Peter et al., 1990b). From these observations it appears that dominant negative effects due to lack of phosphorylation of the COOH-terminal sequences by cdc2 kinase may not be essential for nuclear envelope disassembly/reassembly at mitosis. Although the data suggest that lamin A tail sequences are dis-

pensable they do not rule out the possibility of involvement in cellular processes that may not be evident in mouse L cells.

Similar questions can be asked of the role of the head domain of nuclear lamins. We demonstrated that substitution of the head domain of lamins for that of NF-L did not compromise NF(+La/head) assembly in the cytoplasm. However, it should be noted that NF(+La/head) coassembly with vimentin was weakened, since localization to the nuclear lamina was increased when a NLS was added. The ability of NF(+La/head) to coassemble with vimentin in the cytoplasm (at least as indicated by immunofluorescence staining of filaments) suggests that the size of the head domain may not be critical for assembly. Thus the 34-amino acid head domain of lamin A although considerably smaller in size than that of NF-L (which is 93 amino acids long) can nevertheless integrate into the preexisting vimentin network. These results suggests that the charge and not sequence per se may be important for IF assembly. It will be of interest to determine the pathway of how these chimeric molecules are assembled since lamin assembly is distinct from that of cytoplasmic IF proteins. The first stages of lamin assembly are thought to occur by a head to tail interaction of dimers (Heitlinger et al., 1991; Peter et al., 1991), whereas cytoplasmic IF proteins appear to assemble by lateral association.

A hallmark of lamin staining is rim staining of the nucleus seen by immunofluorescence light microscopy (Gerace et al., 1978). This staining pattern was observed with the chimeric lamin construct pLa(+NF/tail+NLS+CAAX). However, in the absence of the CAAX motif, chimeric La(+NF/tail+NLS) was localized as a meshwork of filaments within the nucleus with a cage-like appearance, but did not have a rim fluorescence by light microscopy. This result suggests an important aspect of nuclear lamin assembly that has not been addressed to date. How does lamin C, which does not contain a CAAX motif, assemble at the nuclear envelope? The chimeric lamin molecule pLa(+NF/tail+NLS) may be expected to behave similar to lamin C since it too does not contain a CAAX motif. Lamin C may therefore have a similar distribution to La(+NF/tail+NLS), a cage-like staining with no rim fluorescence by light microscopy. If correct, this would suggest that lamins A and C may occupy slightly different locations at the nuclear lamina, lamin A being closer to the nuclear membrane while lamin C more interior to the nucleus. Interestingly, Horton et al., (1992) found that when transfected into cells, lamin A was assembled throughout the cell cycle, whereas lamin C assembly was cell cycle regulated. Furthermore, it has been demonstrated that localization of lamin A to the nuclear envelope is regulated by a biosynthetic pathway, of which isoprenylation of the protein appears to be an essential requirement (Horton et al., 1992; Lutz et al., 1992; Sinensky et al., 1994). Conversely, assembly of lamin C in P19 embryonal carcinoma cells was dependent on dissolution of the nuclear envelope (Horton et al., 1992). These differences suggest that the tail domains of lamins A and C are not equivalent in assembly properties, and that the two proteins might be targeted to potentially different locations at the nuclear lamina.

A peculiar property of pLa(+NF/tail+NLS+CAAX) was that it behaved like a lamin B and not like its parent lamin

A. This phenotype was found upon insertion of both a NLS signal and a CAAX motif to the tail of NF-L. The CAAX motif, a target for posttranslation modification, is subsequently removed from mature lamin A by proteolytic cleavage (Weber et al., 1989; Hennekes and Nigg, 1994). In contrast, the CAAX motif of lamin B is not removed. Interestingly, it is only lamin B that remains associated with membrane remnants at mitosis upon nuclear envelope disassembly (Gerace and Blobel, 1980; Gerace et al., 1987). Presumably, association of lamin B with membranes at mitosis is promoted by posttranslational modification (including isoprenylation) of the CAAX motif. The apparent conversion of lamin A to lamin B behavior probably resulted from lack of cleavage of the CAAX motif from the chimeric lamin/NF-L molecule. This supports the model suggested by Hennekes and Nigg (1994) that lamin B may also be anchored to membranes by a similar mechanism.

Curiously many of the cells transfected with the modified IF proteins had abnormal morphology and nuclei which were multinucleated or grossly distorted in shape and structure. At present we have been unable to determine whether the altered NF-L proteins affected either nuclear structure, or caused defects in cell cycle progression. Introduction of the chimeric proteins to cells and monitoring their effects during different stages of the cell cycle will be required to resolve these issues. Furthermore the relationship between localization of chimeric constructs and their assembly with endogenous IF arrays remains to be elucidated. Detailed in vitro assembly using purified IF proteins combined with biochemical fractionation studies of transfected cells will be required to fully understand the mechanisms of IF sorting.

Finally, it should be noted that IF sorting would clearly be influenced by the dynamics of cell cycle changes especially those associated with dissolution of the nuclear envelope at mitosis. Our results do not distinguish effects that result from pre- and postmitotic effects of the cell cycle. Instead they tend to illustrate a static view of the combined effects of protein sorting that manifest during dynamic processes of the cell cycle.

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