

Site-Specific Phosphorylation of the Middle Molecular Weight Human Neurofilament Protein in Transfected Non-neuronal Cells

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We expressed the human mid-sized neurofilament subunit (NF-M) using genomic DNA in mouse L cells and showed that it is transcribed and translated into a protein capable of assembly into the cytoskeleton and of forming a filamentous network that colocalizes with the endogenous vimentin filaments. Moreover, human NF-M expressed in L cells is phosphorylated at sites within the multiphosphorylation repeat (MPR), i.e., the major sites of phosphorylation of NF-M *in vivo*. We also expressed a genomic construct lacking the MPR domain in the native molecule and showed that this MPR(-) protein also was expressed and formed a filamentous network despite diminished incorporation of radiolabeled phosphate. Two major conclusions emerged from the work described in this paper: human NF-M is translated, assembled, and phosphorylated at physiological sites without the need of any other specific neuronal proteins; phosphorylation sites other than the MPR are present within NF-M which may play a role in synthesis, assembly, and degradation of NF protein in humans.

Axons are a unique structural feature of neurons; they are cytoplasmic extensions whose volume and surface area dwarf the dimensions of the cell soma proper. As such, the axon contains many structural features unique to neurons of which perhaps the most striking is the axonal cytoskeleton (for a recent review, see Hollenbeck, 1989). The most abundant proteins within the axons of mammalian neurons are neurofilament (NF) triplet proteins, the primary intermediate filament (IF) system in neurons. NFs are a macromolecular complex comprised of 3 polypeptides designated NF-L, NF-M, and NF-H (low, middle, and high M_r , respectively). The number of NFs and amount of the triplet proteins correlate with the diameter of the axon, and NFs have been proposed to play a part in the regulation of axonal diameter (Hoffman et al., 1984, 1987).

From genomic sequences it is clear that the NF genes belong to a distinct subset, denoted type IV (see Steinert and Roop, 1988, for review of IF structure and nomenclature) within the

IF genes. Type I-III IF genes have 6 introns, which interrupt the coding sequence in exactly the same relative positions, whereas type IV NF genes have only 2 or 3 introns, which are positioned differently from those of the type III genes (except for 1 intron that corresponds to 1 of the type III introns) and those of the other IFs (Lewis and Cowan, 1986; Myers et al., 1987; Lees et al., 1988). While the various IF genes are clearly descendants of a single progenitor gene, the relationship of the NFs to the other IFs is debated (Lewis and Cowan, 1986; Myers et al., 1987).

The coding sequences of the COOH-terminal extensions of the larger M_r NF polypeptides have proven to be of particular interest; the human NF-M gene and the NF-H genes of human, mouse, and rat have been shown to code for multiple repeats (up to 50 for the NF-H genes) based on the sequence KSPV(A) (Myers et al., 1987; Lees et al., 1988; Shneidman et al., 1988). It now appears that the larger M_r NF polypeptides of all species examined (except for rat and mouse NF-M, which have single sequence motifs resembling the one found in human NF-M) have multiple repeats based on the sequence KSPV(A) (Levy et al., 1987; Myers et al., 1987; Napolitano et al., 1987; Pleasure et al., 1989; and unpublished observations). The repeat motif in human NF-M is a sequence of 13 amino acids (aa), i.e., KSPVPKSPVVEEKG repeated serially almost exactly 6 times (Fig. 1). This repeated sequence of 13 aa in human NF-M contains 2 KSPVs with the serines at position 2 and 7 (see Fig. 1). Using monoclonal antibodies (mAbs) that recognize various phosphoisoforms of human NF-H and NF-M and chemically phosphorylated peptides based on the KSPV(A) motif, the regions containing these repeats have been shown to be the major site of NF phosphorylation which may be responsible for generating the extensive heterogeneity of phosphorylation found in NF *in vivo* (Lee et al., 1988a, b). The repeat motifs have thus been called the multiphosphorylation repeat (MPR) (Lee et al., 1988a, b). Recent work in our laboratory has shown that both serines in the 13 aa repeated sequence of human NF-M are sites of phosphorylation (Tangoren et al., 1989; and I. Tangoren, L. Otvos, and V. M.-Y. Lee, unpublished observations) and that the single repeat motif in rat NF-M also is phosphorylated (Tangoren et al., 1989; Xu et al., 1989). The functional significance of the phosphorylation of the MPR regions in NFs is unknown, although there is correlative evidence in the lamprey to show that it may be involved in determining axonal diameter (Pleasure et al., 1989) or in mammals that it may somehow control the rate of slow axonal transport (Carden et al., 1987; reviewed by Matus, 1988). Also, little is known about the identity of

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possible sites of phosphorylation in NF-M outside the MPR that may play a role in regulating assembly, as has been shown for other IFs (Inagaki et al., 1987). Nevertheless, sites of phosphorylation outside the MPR are thought to exist based on the specificity of phosphorylation-dependent mAbs that do not recognize the MPR, and these putative sites have been postulated to play a role in structural functions of lamprey NF (Pleasure et al., 1989).

In this report we demonstrate that genomic clones of human NF-M are expressed in mouse L cells, and that they are translated and assembled into filaments without the presence of any other neuronal proteins, confirming another recent report (Monteiro and Cleveland, 1989). More important, we show that human NF-M can be expressed from genomic DNA and is phosphorylated within the MPR in transfected L cells. This represents the first report that NFs, the most abundant class of neuronal structural proteins and protein kinase substrates of neurons, can be phosphorylated appropriately in non-neuronal cells. Finally, we have also expressed a construct lacking the MPR in L cells to determine whether the MPR is essential for human NF-M expression and assembly, and whether phosphorylation sites other than those comprising the MPR exist in human NF-M.

Materials and Methods

Construction of plasmids and transfection of L cells

pTZNFM. A 6.6 kb Bam HI to HindIII fragment was isolated from a lambda phage clone and ligated into pTZ18 (Fig. 1). This fragment contains the entire human NF-M gene and 2.5 kb of upstream sequence. The resulting plasmid was used directly for transfection experiments.

pTZNFM Δ Bam-. This plasmid was derived from pTZNFM by removing a 726 bp Bam HI to Bam HI fragment from the third exon. This creates an in-frame deletion removing amino acids 552–793.

DNA transfection. One and one-half microgram of construct plasmid was mixed with 15 μ g of selectable plasmid [either pSV₂neo (Southern and Berg, 1982) or pTK11 (Bradshaw and Deininger, 1984)] in a solution containing 5 gm/liter HEPES pH 7.10, 8 gm/liter NaCl, 0.37 gm/liter KCl, 1 gm/liter glucose, and 0.095 gm/liter Na₂HPO₄, and precipitated by addition of CaCl₂ to 125 mM. This calcium phosphate coprecipitate was added to murine L TK-cells (RJK 691) in 1 ml/10 cm dish at 1/3 confluence. Cells were grown 48 hr without selection, split 1:10 into either HAT (pTK11) or 400 μ g/ml G418 (pSV₂neo), and cloned or collected at 10–14 d posttransfection. Efficiencies ranged from 1 to 100 colonies per microgram of selectable plasmid.

Northern analysis of NF expression in cells

Poly A⁺ RNA was isolated using a kit purchased from Invitrogen which was used exactly according to the manufacturer's specifications from cells in log phase growth. RNAs were analyzed by electrophoresis through 1% agarose gels containing formaldehyde (Thomas, 1980) and transferred to nylon membranes (Zeta-Probe/BioRad). Hybridization was to a partial cDNA clone of human NF-M (pHNF₂; Myers et al., 1987) in 50% formamide, 6 \times SSPE, 1 \times Denhardt's, 0.1% SDS, and 100 μ g/ml salmon sperm DNA. Posthybridization washes were 1 \times with 2 \times SSC/0.1% SDS at room temperature, and 1 \times with 0.2 \times SSC 0.1% SDS at 58°C. Exposure was as stated in the legend of Figure 2.

Preparation of mAbs and antiserum

The mAbs used in this study were prepared and initially characterized previously (Carden et al., 1985; Lee et al., 1987).

The antivimentin antiserum was prepared by immunizing rabbits with a 22 amino acid synthetic peptide representing the sequence of amino acids 438–459 from the carboxyl terminal region of human vimentin (Ferrari et al., 1986). The antiserum derived from these rabbits was used at a dilution of 1:500 for immunofluorescence or immunoblotting.

Indirect immunofluorescence

Cells were grown on glass coverslips for at least 48 hr, after which they were fixed by immersion in –20°C acetone for 7–10 min and allowed to air-dry. The coverslips were then incubated with the primary mAb for 1 hr at room temperature in a humidified chamber. The coverslips were then washed 4 times for 15 min in PBS and incubated with the secondary Ab (goat anti-mouse coupled to FITC and goat anti-rabbit coupled to RITC; Cappel) for 1 hr at room temperature in a humidified chamber, after which they were washed again 4 times for 15 min in PBS and mounted with aquamount.

Preparation of cytoskeletal extracts and total cell extracts

Confluent 60 mm plastic tissue culture dishes were lysed with 200 μ l of cold Tris buffered saline (TBS)/Triton with a cocktail of protease inhibitors added and scraped into a cold glass homogenizer. The lysate was incubated for 30 min on ice with intermittent plunges of the homogenizer. The insoluble cytoskeleton was pelleted at 100,000 \times g in a Beckmann TL100 centrifuge and the pellet solubilized in Laemmli sample buffer (LSB) without dye for protein determination using a Pierce protein assay kit dependent on Coomassie blue dye binding. Total cell extracts were prepared by homogenizing cells in boiling LSB without dye and boiling the extract for 15 min.

SDS-PAGE and immunoblotting

Cell extracts were separated on 0.75-mm-thick 4–8% gradient or 7.5% polyacrylamide gels exactly as described previously (Lee et al., 1987). Proteins separated by SDS-PAGE were electrophoretically transferred to either nitrocellulose (Schleicher and Schuell) or Immobilon-P (Millipore) and probed with mAbs and were visualized using the peroxidase-antiperoxidase (PAP) protocol as described (Lee et al., 1987).

Quantitative immunoblotting

Pure bovine lens vimentin was purchased from Boehringer Mannheim. Bovine NF-M which was judged to be >95% pure was purified by anion exchange chromatography on a DEAE column from neurofilament triplet proteins isolated according to Carden et al. (1985). Various amounts of cytoskeletal extracts were separated on 7.5% SDS-PAGE gels and blotted to nitrocellulose. Included on these gels were known amounts of the appropriate standards, vimentin or NF-M, which were used to generate standard curves for each antigen. The replicas were incubated overnight with the primary Abs (either RMO 189, an anti-NF-M core mAb, or our rabbit antivimentin antiserum). Following washes the replicas were incubated with 1 \times 10⁶ cpm/ml of either anti-mouse IgG labeled with ¹²⁵I or Protein A labeled with ¹²⁵I. The replicas were washed and exposed to film. The films were scanned with an LKB ultrascan laser densitometer and standard curves computed for each film.

[³²P]PO₄ and [³⁵S]-methionine labeling and immunoprecipitations

Confluent 60 mm plastic tissue culture dishes of cells were washed once with RPMI 1640 phosphate-free medium (Gibco) containing 10% fetal bovine serum (FBS; JR Scientific), 2 mM L-glutamine, 10,000 units/ml penicillin, and 10 mg/ml streptomycin and then phosphate-starved by incubation in the phosphate-free medium for 15 min at 37°C; 2.5 mCi of [³²P]-orthophosphate (Amersham) was added to each culture dish and the cells were labeled for 2 hr at 37°C. For pulse chase experiments confluent dishes of MNA and MNA-B were starved in methionine-free RPMI 1640 medium for 20 min and then labeled for 2 hr with 1.25 mCi of trans-[³⁵S]-label (ICN) per dish and chased with complete medium for 0 or 18 hr, as indicated in Figure 6. Immunoprecipitations were conducted as described in Black and Lee (1988). After separation, the gels were dried and exposed to film for times stated in the figure legends.

The pulse chase experiment in Figure 6 represents half of a gel where duplicate samples were processed from the same plates of cells. Densitometric values represent the average of the duplicate sets which were scanned on an LKB ultrascan densitometer.

Dephosphorylation of L-cell cytoskeletal extracts

L-cell extracts were dephosphorylated with *Escherichia coli* alkaline phosphatase (Sigma type III) using 4 units/mg of protein exactly as described by Carden et al. (1985). The reaction was allowed to proceed

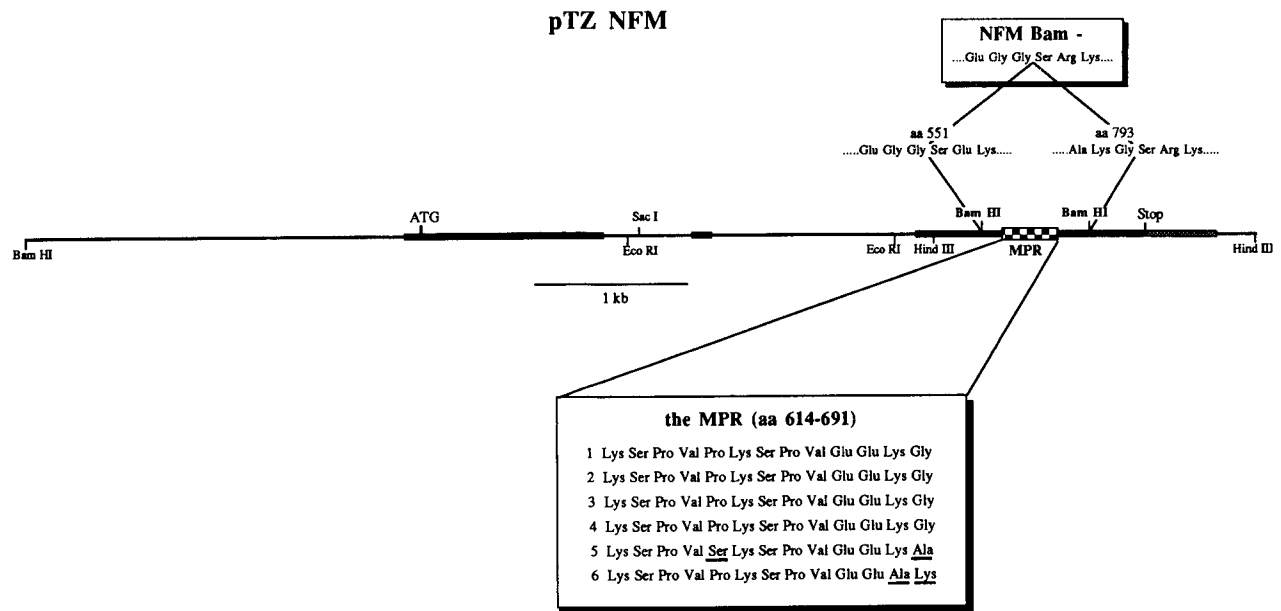


Figure 1. Schematic representation of the human NF-M gene and the constructs used for transfection, including the relative position of molecular structures discussed in this paper within the NF-M protein. The MPR (and its full aa sequence, from Myers et al., 1987), the sites of Bam HI cleavage, and the aa sequences juxtaposed by this deletion are indicated.

at 37°C for 16 hr. Control extracts were treated in exactly the same way except that no enzyme was added.

Two-dimensional gel analysis

Iso-electric focusing was performed according to the method of O'Farrell exactly as described previously (Pleasure et al., 1989). A pH gradient of 4.5–8.0 was achieved using LKB ampholines (pH 3.5–10.0, 5.0–7.0, and 4.0–6.0 in a ratio of 2:9:9 at a final concentration of 2%); 20 µg of a cytoskeletal extract prepared from confluent 60 mm dishes of both MNA and MNA-B cells were loaded on the first dimension. The second dimension consisted of 4–8% gradient SDS-PAGE gels.

Results

Transfection and expression of human NF-M constructs

L cells generated by transfection with the full-length genomic clone for human NF-M are designated as MNA cells, while those generated by transfection with the Bam HI digested clone [i.e., MPR(–)] are designated as MNA-B cells (Fig. 1). After transfection and selection, the cells were cloned by limiting dilution, and multiple clones were examined by Northern blotting, indirect immunofluorescence, and/or Western blotting for NF-M mRNA and protein expression, respectively. Several clones were isolated which expressed each of the 2 constructs; typical Northern blot results are shown in Figure 2. NF-M message is clearly visible in the lane containing MNA RNA, which comigrates exactly with the authentic NF-M mRNA isolated from human brain (data not shown; Myers et al., 1987). In addition, we determined that the endogenous murine NF genes encoding NF-L, NF-M, or NF-H were not induced (data not shown). The expression of NF-M in fibroblasts transfected with this construct implies that the tissue-specific expression of NF-M is not controlled exclusively in *cis* within the 3 kb of upstream elements included in this construct or that NF-M expression is deregulated by the presence or absence of some other factor(s). In addition, Figure 2 shows that NF-M lacking the Bam HI restriction fragment is capable of expression in L cells and that the stably transfected MNA-B cells make mRNA that is ap-

proximately 2500 bp in size. This reflects the removal of 726 bp from the MPR(–) construct. The second, more rapidly migrating band in both lanes has been observed before in human brain RNA (Myers et al., 1987) and in RNA isolated from some human neuronal tumor cell lines (unpublished observations). Its origin is unknown but it may be due to differential polyadenylation signals or splicing in the 3' end of the mRNA. The levels of MPR(–) NF-M mRNA in MNA-B cells are significantly lower than the amount of the full-length mRNA in MNA cells. This finding may explain the reduced levels of MPR(–) NF-M protein expression in MNA-B cells when compared to the amount of full-length NF-M protein expression in MNA cells (see below and Fig. 3). The reason for the reduced expression of the MPR(–) NF-M mRNA and protein is not known, but it is most likely a clonally related phenomenon because other clones expressing the NF-M constructs described here have varying levels of protein and mRNA expression. It is unlikely that the difference in NF-M and MPR(–) NF-M mRNA expression is due to differential stability of the 2 messages because the region deleted from MPR(–) NF-M is wholly exonic and would not be predicted to make a great change in either splicing or mRNA conformation.

Whole cell extracts were prepared from MNA and MNA-B cells and probed using a library of anti-NF mAbs (Carden et al., 1985; Lee et al., 1987, 1988a) to determine if NF-M and MPR(–) NF-M were being translated (Table 1). All mAbs that reacted with human NF-M isolated from human spinal cord reacted with the full-length NF-M in MNA cells, suggesting a close similarity between authentic human NF-M and human NF-M expressed in the MNA cells. All of the MPR-specific mAbs which have been shown to react with the MPR in human NF-M did not react with the MPR(–) NF-M in the MNA-B cells. All mAbs known to be specific for core epitopes in NF-M and all mAbs specific for the heptad repeat region at the extreme carboxy terminal region of NF-M (unpublished observations) were positive for extracts from both MNA and MNA-B cells.

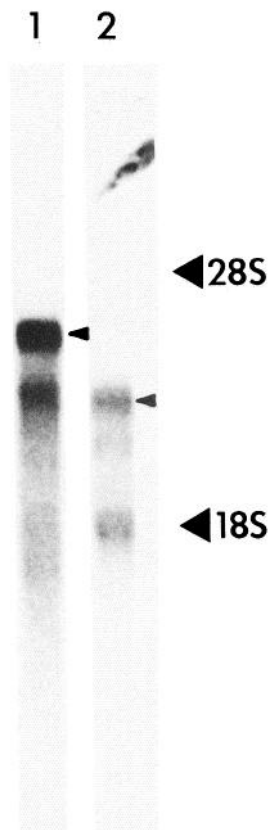


Figure 2. Northern hybridization of RNAs (4 d exposure) from transfected cell lines. Poly A⁺ RNA from transfected cell lines were hybridized with a ³²P α dCTP labeled NF-M probe (pHNF 4.2; Myers et al., 1987). Lane 1, 2 μ g MNA Poly A⁺ RNA. Lane 2, 10 μ g MNA-B Poly A⁺ RNA. The lower M_r band seen in lane 2 (arrowhead) represents transcription from the transfected MPR(-) NF-M construction. Its length is appropriate for the removal of the 726 bp Bam HI fragment. The positions of 28S and 18S RNAs are indicated as markers of molecular weight. Arrowheads denote the bands comprising the major NF-M mRNA species.

This indicates that intact NF-M and MPR(-) NF-M are both translated into protein and that the MPR-specific mAbs are incapable of detecting NF-M following the removal of the MPR domain. In contrast, mAbs specific for structural determinants coded for by sequences both 5' and 3' of the deletion are found in MPR(-) NF-M.

Included among the MPR-specific mAbs that detect full-length NF-M are those dependent on the state of phosphorylation of

the MPR (Lee et al., 1988a, b). A number of these mAbs have previously been defined as P⁻, Pind, or P⁺ according to their susceptibility to the removal of phosphates (P) using alkaline phosphatase to dephosphorylate NF-M (P⁻ mAbs are those mAbs which react with native human NF only after alkaline phosphatase treatment, P⁺ are those which react with native human NF only before alkaline phosphatase treatment, and Pind are those which react with native human NF before and after alkaline phosphatase treatment; see Lee et al., 1987, 1988a, b, for further descriptions of the classification of anti-NF-M mAbs). Members of all of these groups of mAbs are included in Table 1. These groups of mAbs react with the full-length NF-M protein in transfected MNA cells (see Fig. 7 also), indicating that NF-M is likely to be phosphorylated at the MPR to some degree in MNA cells.

NF-M and MPR(-) NF-M are incorporated into the cytoskeleton of transfected L cells

Triton X-100 insoluble fractions of MNA and MNA-B cells were prepared to determine if human NF-M is incorporated into the cytoskeleton of the L cells. Figure 3 shows nitrocellulose replicas of SDS-PAGE gels probed with RMO 189, a mAb specific for a core determinant in NF-M (Fig. 3A), and an antiserum raised to a synthetic peptide based on the sequence of human vimentin (Fig. 3B). It is clear from the data shown in this figure that, although the same amount of protein was loaded in each lane, NF-M and MPR(-) NF-M are greatly enriched in the Triton-insoluble lanes derived from MNA and MNA-B cells. Furthermore, virtually no immunoreactivity was found in the soluble fraction (data not shown), suggesting that both NF-M and MPR(-) NF-M are incorporated into the cytoskeleton of these transfected L cells. The immunoband in the lanes containing the MNA-B protein extracts has an apparent M_r of 100 kDa (Fig. 3A) despite the removal of coding material which should account for only a 25 kDa change in the full-length 170 kDa protein. Considering previous data that have shown that the migration of NF is highly dependent on phosphorylation (Carden et al., 1985), and that the human NF-M mRNA codes for a protein with a M_r of only about 102 kDa (Myers et al., 1987), this observation may in part reflect the removal of the majority of the sites of phosphorylation from NF-M. The MPR(-) NF-M still is retarded in its SDS-PAGE mobility when its true M_r is considered. This is probably due to the numerous glutamic acid residues in the COOH terminal extension of NF-M. In addition, immunoreactive full-length NF-M is distributed in M_r 's ranging from 150 to 165 kD, suggesting possible heterogeneity due to different states of phosphorylation at the MPR

Table 1. Reactivity of anti-NF mAbs with transfected NF-M proteins

	Full-length NF-M	MPR(-) NF-M	Representative examples of mAbs
Known mAbs specific for MPR	57	0	RMO 308, RMdO 20, and HO 45
Known mAbs specific for core	53	53	RMO 189
Known mAbs specific for the sidearm outside MPR	3	2	RMO 3
Known mAbs specific for carboxy terminal heptad repeats	6	6	RMO 270

This table summarizes the results of immunoblots of cell extracts from L cells expressing full-length NF-M or MPR(-) NF-M with anti-NF mAbs. Over 500 mAbs were screened of which all (233) that were positive for NF-M isolated from human spinal cord were also reactive with full-length NF-M from L cells.

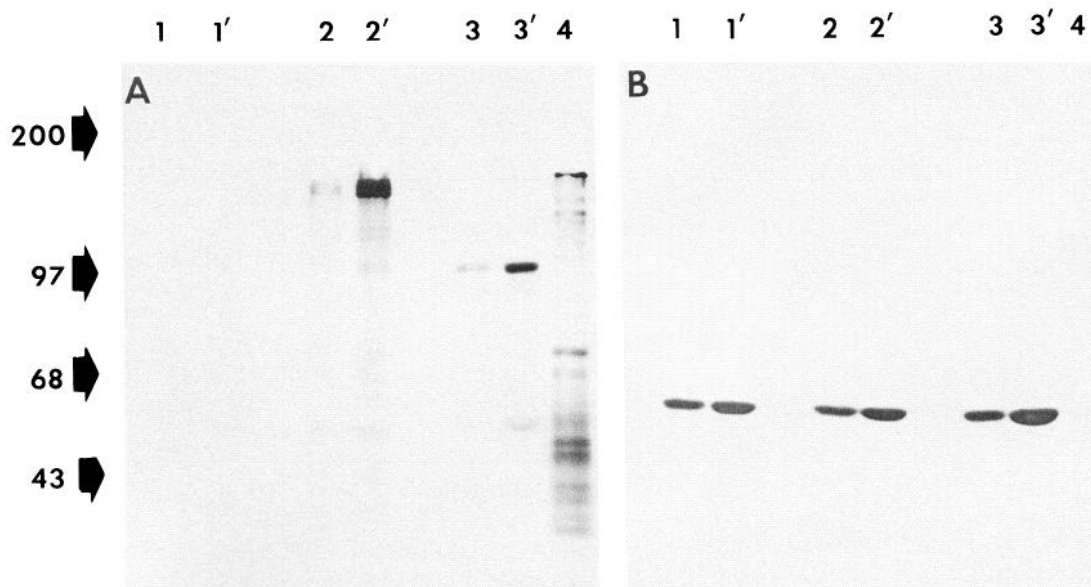


Figure 3. Association of full-length NF-M and MPR(-) NF-M with the cytoskeleton of transfected L cells. Shown are immunoblots derived from 1-dimensional SDS-PAGE (7.5% acrylamide) which compare the relative amounts of NF-M and vimentin in total cell extracts vs. Triton X-100 insoluble extracts. *A*, Immunoblot probed with RMO 189, an anticore NF-M mAb; *B*, immunoblot probed with antivimentin antiserum. Total cell extracts (30 μ g of protein) from untransfected L cells (lanes 1), MNA (lanes 2), and MNA-B (lanes 3) were compared to Triton-insoluble fractions (30 μ g of protein) from untransfected L cells (lanes 1'), MNA (lanes 2'), and MNA-B (lanes 3'). Lane 4 contains NF isolated from human spinal cord (which is somewhat degraded due to the postmortem interval). *M_r*'s are shown at the left in kDa.

(see below). Since MPR(-) NF-M protein migrates as a single band, this implies that almost all of the heterogeneity due to phosphorylation at the MPR has been lost. Figure 3*B* shows that there is no apparent change in the levels or solubility of the endogenous vimentin protein from the L cells. Quantitative immunoblotting of extracts of MNA cells using an antivimentin antiserum or the anticore NF-M mAb (RMO 189), showed that vimentin constitutes 21.9% (SD 3.03) and NF-M 1.6% (SD 0.07) of the total Triton X-100 insoluble protein (data not shown).

Cellular localization of the NF-M and MPR(-) NF-M proteins

Indirect immunofluorescence studies using anti-NF mAbs on the MNA and MNA-B cells revealed that both the full-length and the MPR(-) NF-M stained in a filamentous manner. RMO 308, an MPR-specific mAb, clearly reacted with an abundant network of filaments containing the full-length NF-M, and these filaments colocalized exactly with endogenous vimentin. This suggests that both vimentin and NF-M proteins are incorporated into the IFs of MNA cells (Fig. 4, *A, B*). RMO 3, an mAb specific for an epitope outside the MPR in the sidearm of NF-M, stained MPR(-) NF-M in MNA-B cells, and this immunoreactivity also colocalized with vimentin (Fig. 4, *C, D*). However, the intensity and the distribution of MPR(-) NF-M were reduced compared to the full-length NF-M protein or vimentin. The reason for this reduction is not known at present but it may be due to the lower levels of MPR(-) NF-M mRNA rather than to a decrease in the stability of the MPR(-) NF-M protein (see below). Finally, as expected from the foregoing, RMO 308 did not stain MNA-B cells (Fig. 4*E*) nor did any of the anti-NF mAbs stain untransfected L cells (data not shown).

The pattern of staining for NF-M in MNA cells was noteworthy because the most abundant NF immunoreactivity typically was clustered around the nucleus and radiated outward

toward the cell periphery (Fig. 4*A*). A minority of the MNA cells adopted a flat morphology with extended cytoplasm. Occasionally multiple nuclei were noted in these cells. This morphology also was seen in untransfected L cells with the same frequency. The transfected flat cells usually stained intensely both with anti-NF mAbs and the antivimentin antiserum. In addition, occasional cells had primarily a perinuclear whorl of anti-NF immunoreactivity which colocalized with vimentin immunoreactivity. This pattern of staining may represent the previously observed site of assembly of soluble IF subunits into the IF network (Vikstrom et al., 1989). That full-length NF-M is present at approximately $\frac{1}{4}$ the level of vimentin in the transfected MNA cells, yet colocalizes extensively with vimentin, is strong evidence in favor of the notion that vimentin and NF-M are assembled into the same IFs or that IFs comprised of either NF-M or vimentin interact significantly.

We have used our cell transfectants to determine the specificity of some anti-NF mAbs for other subdomains of NF-M and to attempt to characterize their structural role in NF-M. For example, anticore NF-M mAbs (such as RMO 189) did not immunostain either NF-M or MPR(-) NF-M. In fact, of 53 anti-NF-M core mAbs used in these indirect immunofluorescence studies, none were reactive with the NF-M or MPR(-) NF-M. This contrasts with several other cell lines (including PC 12 cells) in which the neurofilament triplet is expressed and anti-NF-M core mAbs stain in a filamentous array (unpublished observations). This implies that the core epitopes recognized by these mAbs are masked *in situ*, reflecting differences in antigen presentation brought about by the probable coassembly of NF-M with vimentin. Finally, several mAbs specific for the heptad repeat region at the carboxy terminal of NF-M, when tested on MNA and MNA-B cells, all stained both full-length NF-M and MPR(-) NF-M (Table 1 and data not shown). These data further indicate the integrity of the product of the deleted construct.

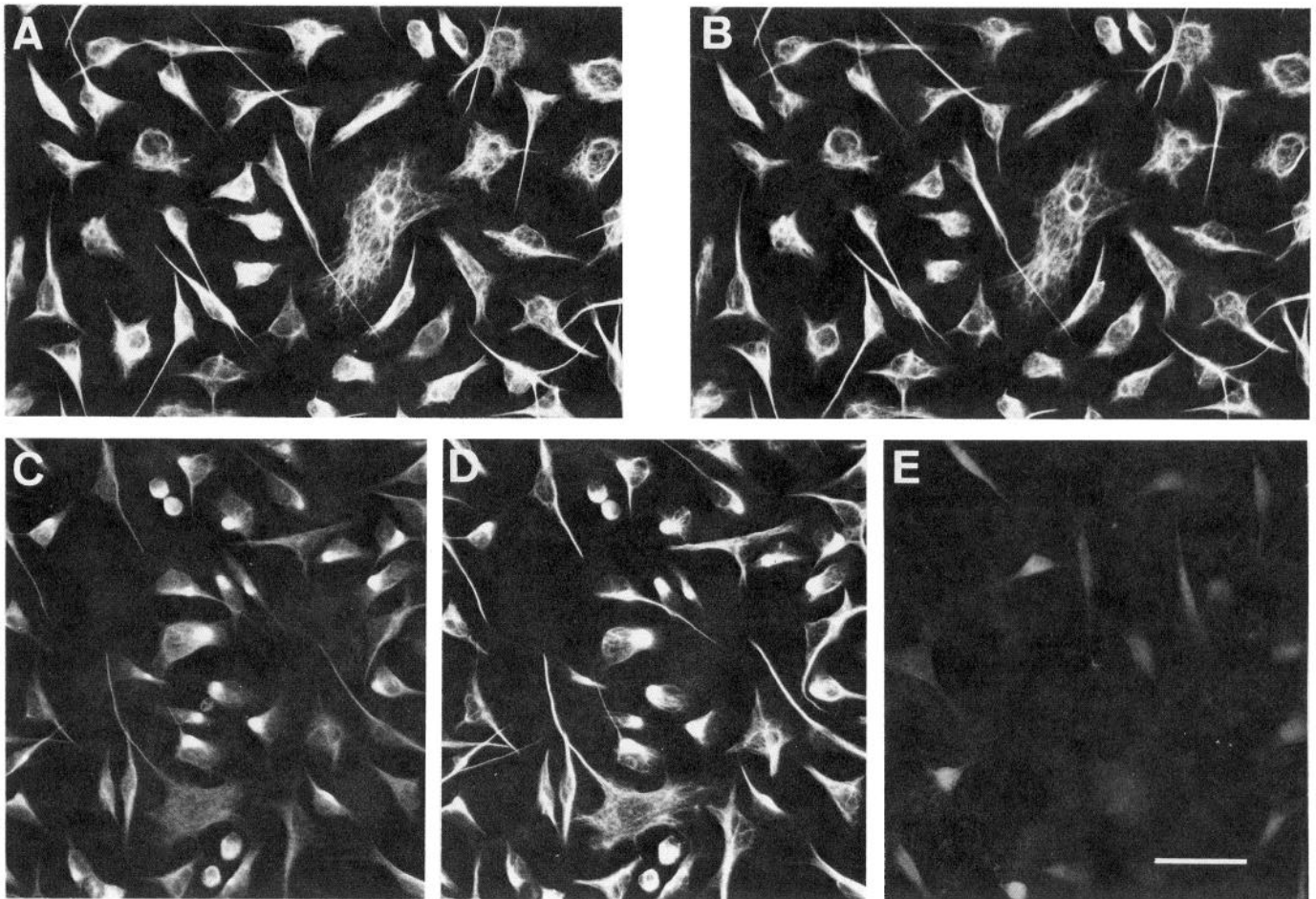


Figure 4. Immunofluorescent localization of full-length NF-M and MPR(-) NF-M proteins in transfected L cells. *A* and *B*, MNA cells double-stained with RMO 308 (*A*; visualized by FITC coupled anti-mouse second Ab) and rabbit antivimentin (*B*; visualized by RITC coupled anti-rabbit second Ab). *C* and *D*, MNA-B cells double-stained with RMO 3 (*C*; visualized by FITC-coupled anti-mouse second Ab) and rabbit antivimentin (*D*; visualized by RITC coupled anti-rabbit second Ab). *E*, MNA-B cells stained with RMO 308. The photograph in *E* was exposed approximately 3 times longer than those in *A–D*, which accentuates the slight background nuclear staining in some cells. Scale bar = 4 μ m.

Stability of full-length NF-M and MPR(-) NF-M proteins

Pulse-chase experiments were conducted to examine whether the limited distribution and decreased amount of MPR(-) NF-M in MNA-B cells might be due in part to decreased stability of the grossly altered protein product. When MNA cells were labeled with [³⁵S]-methionine and chased for 18 hr, approximately 39% of the labeled full-length NF-M remained after the chase (Fig. 5, lanes 1 and 1'). A similar result (38%) was found when MNA-B cells were used. However, it is clear that there is a smaller amount of labeled MPR(-) NF-M than of the full-length protein (Fig. 5, compare lanes 1 and 2). Labeled MPR(-) NF-M at 0 hr constituted 40% of full-length NF-M at 0 hr. We conclude that the difference in intracellular distribution of the full-length and deleted products was not due to decreased stability of MPR(-) NF-M, but instead may reflect decreased synthesis of the protein, and that the removal of the MPR has no effect on the stability of human NF-M.

Both NF-M and MPR(-) NF-M are phosphorylated

Two-dimensional gel analysis of a mixture of cytoskeletal extracts from MNA and MNA-B cells contributes further evidence

that the removal of the MPR greatly simplifies the pattern of phosphorylation of NF-M in these cells and that the M_r heterogeneity of the full-length protein is due to phosphorylation. Silver-stained gels show the relative positions of the 2 NF-M proteins among the large number of proteins in the cytoskeletal extracts (Fig. 6*A*). Probing nitrocellulose replicas of these 2-dimensional gels with RMO 189, a core-specific anti-NF-M mAb, elicited a complex pattern of immunoreactivity (Fig. 6*B*). A streak of immunoreactivity stretching from a more basic isoelectric point (pI) with a M_r of 150 kDa to a more acidic pI with a M_r of 165 kDa was detected together with a single spot at 100 kDa. In contrast, when similar immunoblots were probed with RMO 308, an MPR-specific anti-NF-M mAb, only the streak at 150–165 kDa was seen (Fig. 6*C*). We conclude that the streak at 150–165 kDa is full-length NF-M whereas the spot at 100 kDa is MPR(-) NF-M. This conclusion is also supported by experiments in which cytoskeletal extracts were treated separately (data not shown). Further, we suggest that the streak may represent the sequential addition of phosphate to the full-length NF-M, resulting in isoelectric variants with more acidic pIs and slower electrophoretic mobilities. That the pI of the MPR(-) NF-M is not very different from that of the full-length NF-M

may be due to the large number of glutamic acid residues remaining in the sidearm of the truncated NF-M (Myers et al., 1987).

MNA and MNA-B cells were metabolically labeled *in vivo* with [32 P]PO $_4$ and immunoprecipitated with anti-NF-M mAbs to determine whether phosphates are incorporated into NF-M and MPR(-) NF-M in these cells. Figure 7 shows typical results for these experiments. RMO 189 (an anticore NF-M mAb) and RMdO 20 (an MPR-specific mAb) both immunoprecipitated abundant 32 P-labeled full-length NF-M, indicating heavy phosphorylation of NF-M in these cells. In contrast, immunoprecipitation of MNA-B cells with the same mAbs showed that RMO 189, but not RMdO 20, immunoprecipitated a faintly 32 P-labeled band migrating at 100 kDa. These results indicate that kinases capable of phosphorylating at least 2 distinct sets of sites in NF-M are present in transfected L cells.

Although full length NF-M in L cells is heavily phosphorylated at the MPR, the faster mobility and heterogeneous banding pattern of the full-length NF-M protein expressed in MNA cells when compared to that isolated from human spinal cord indicates that NF-M in these cells exists in a number of phosphoisoforms. Furthermore, close examination shows that only a small amount of the NF-M in these cells comigrates with fully phosphorylated NF-M as isolated from human spinal cord (compare lanes 2 and 3 with lane 4 in Fig. 3 and see Fig. 7). This was confirmed by immunoblotting with P $^+$ mAbs, i.e., those like HO 45 which recognize the highly phosphorylated isoforms of NF-M. These blots revealed that only the most slowly migrating portion of the broad NF-M immunoreactive band is visualized (Fig. 8). Dephosphorylation of L-cell extracts with alkaline phosphatase abolished the immunoreactivity of NF-M with HO 45, proving that HO 45 immunoreactivity was indeed due to phosphorylation. RMO 189, a phosphorylation-independent or Pind mAb, shows that the full range of NF-M MW isoforms is reduced to a single band following dephosphorylation (Fig. 8), implying that the M_r heterogeneity of NF-M in the L cells is due to phosphorylation. The same single band that is present with the Pind mAb after dephosphorylation is the major species which reacts, under all conditions, with RMdO 20, a dephosphorylation dependent, or P $^-$ mAb, which binds to an epitope within the MPR (Fig. 8). This suggests that a significant proportion of the NF-M expressed by the L cells is hypophosphorylated within the MPR. The fact that all of our phosphorylation-dependent, MPR-specific mAbs reacted with full-length NF-M but not MPR(-) NF-M extracted from transfected L cells (Table 1), together with the evidence presented in Figures 7 and 8, supports the conclusion that the MPR which contains the major sites of phosphorylation of human NF-M *in situ* also makes up the major sites of phosphorylation of NF-M in L cells. Finally, the presence of a lightly labeled MPR(-) NF-M species in MNA-B cells further indicates that there are one or more sites of phosphorylation in human NF-M other than the MPR.

Discussion

This report demonstrates for the first time the site-specific phosphorylation of an IF gene product and of a neuronal cytoskeletal protein in non-neuronal cells. Human NF-M was shown to be phosphorylated at the MPR, the region of the molecule that is extensively phosphorylated *in vivo* and may be a determinant of NF function in humans (Lee et al., 1988a, b). In addition, the existence of phosphorylation site(s) outside the MPR was

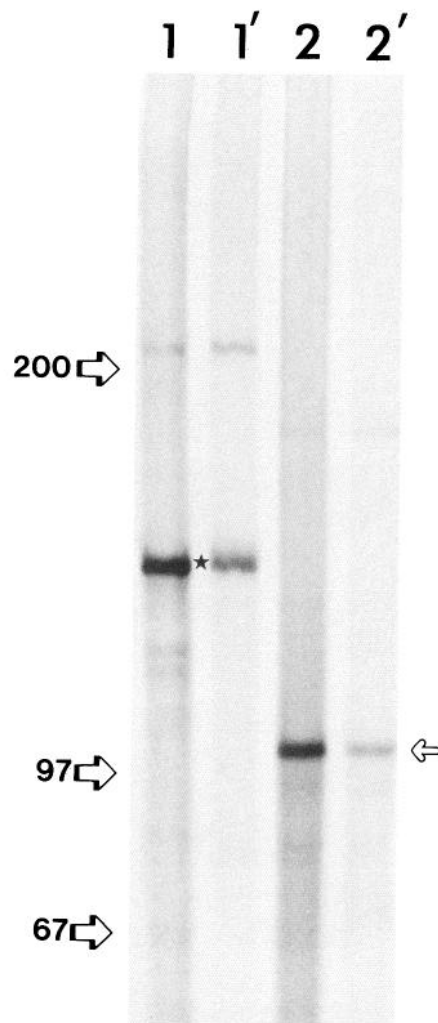


Figure 5. Pulse chase of MNA and MNA-B cells. Lane 1, MNA cells chased for 0 hr. Lane 1', MNA cells chased for 18 hr. Lane 2, MNA-B cells chased for 0 hr. Lane 2', MNA-B cells chased for 18 hr. All samples were immunoprecipitated with RMO 189. The position of full-length NF-M from the L cells is indicated by a star and that of MPR(-) NF-M by arrows. Relative mobilities of M_r standards are represented in kDa at the side of the figure. This figure represents 1 set of 2 duplicates from the same experiment.

also demonstrated in MPR(-) NF-M. Thus, cells transfected with the MPR(-) NF-M, i.e., the MNA-B cells, will be a useful resource to assess the structure and function of other phosphorylation site(s) in human NF-M.

Site-specific phosphorylation of human NF-M within the MPR was confirmed by a variety of criteria. First, mAbs specific for phosphorylated isoforms of human NF-M recognize the full-length NF-M protein in the MNA cells on Western blots (Table 1, Fig. 8). Second, 2-dimensional gel analysis showed that the slower migrating isoforms of NF-M are accompanied by corresponding acidic shifts in pI consistent with increasing levels of phosphorylation within the MPR. Third, NF-M in the MNA cells incorporated [32 P]PO $_4$ which was immunoprecipitated with mAbs specific for phosphorylated epitopes within the MPR (Fig. 7 and data not shown). Finally, dephosphorylation of human NF-M in extracts of the MNA cells reduced the heterogeneity of NF-M isoforms to a single band. This dephosphorylated NF-M was detected by MPR-specific P $^-$ mAbs but not by MPR-spe-

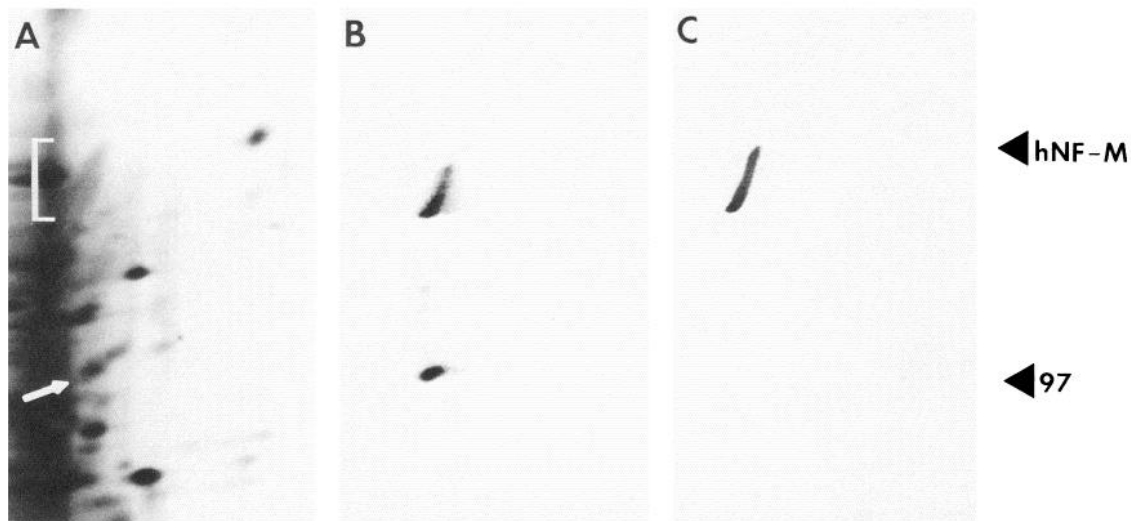


Figure 6. Two-dimensional (2-D) gel analysis of cell extracts from NF-M transfected cells. *A*, Silver stain of a 2-D gel with the positions of full-length NF-M (bracket) and MPR(-) NF-M (arrow) indicated. *B*, Immunoblot of gel identical to that from *A* which has been probed with RMO 189, which recognizes a core epitope found in both full-length NF-M and MPR(-) NF-M. *C*, Immunoblot probed with RMO 308, which recognizes an epitope within the MPR and thus does not see MPR(-) NF-M. The position of NF-M isolated from human spinal cord (approx. 170 kDa) and the 97 kDa M_r marker are shown at the right. The acidic end of the 2-D gels is to the right and the second dimension is SDS-PAGE (4–8% gradient).

cific P⁺ mAbs. However, NF-M in MNA cells was not as extensively phosphorylated as NF-M isolated from human nervous tissue. This was evident from the slightly decreased apparent M_r on SDS-PAGE gels of NF-M in MNA cells when compared with NF-M from human spinal cord (cf. lanes 2 and 2' with lane 4 in Fig. 3) as well as by the abundance of NF-M isoforms which reacted with mAbs specific for dephosphorylated or poorly phosphorylated epitopes (P⁻) in the MPR (Table 1, Fig. 8). This is in sharp contrast with previous studies which showed that human NF-M isolated from human tissue and probed *in situ* by immunocytochemistry exists primarily in the most highly phosphorylated form (Schmidt et al., 1987). This discrepancy may be explained by the fact that L cells, unlike normal neurons, undergo mitosis continually when maintained in 10% fetal bovine serum. Indeed, it will be interesting to study changes in NF assembly and phosphorylation in a mitotic system and compare these features to developing systems where NF-M immunoreactivity is found before the last round of cell division (Tapscott et al., 1981). Like L cells, such developing systems lack the extensively phosphorylated isoforms at this stage (Carden et al., 1987).

Another possible explanation for the relative paucity of the highly phosphorylated forms of human NF-M in transfected L cells may be that the entire array of kinases and phosphatases responsible for the reversible phosphorylation of NF-M within and/or outside the MPR sites is not present or active in L cells. One potential kinase is the one described by Wible et al. (1989), which is highly active on the MPR-containing NF subunits. This kinase is particularly interesting because it phosphorylates partially dephosphorylated bovine NF-H better than extensively dephosphorylated NF-H (Wible et al., 1989). Thus, it is possible that this putative neuron-specific kinase fully phosphorylates the MPR subsequent to initial phosphorylation events performed by more ubiquitous kinases. Our L cells transfected with genomic NF-M DNA will be useful for studies of the interaction between human NF-M and the NF-specific kinase mentioned above and would be an ideal cell line to express this kinase once

it has been cloned. Finally, differences in the phosphorylation state of NF-M in the L cells compared with NF-M isolated from human spinal cord may be due to the lack of other differentiated neuronal characteristics in the L cells (e.g., neurite extension), the regulation of which also might require the expression of similar kinases and phosphatases.

We have also successfully expressed MPR(-) NF-M in L cells by removing a Bam HI restriction fragment containing the MPR encoding sequences from the full-length genomic NF-M construct (Figs. 1, 2). Several lines of evidence presented here demonstrate that the MPR(-) NF-M is translated and has characteristics of the expected deletion product.

Our observation that MPR(-) NF-M is recovered from the detergent-insoluble cytoskeleton and colocalizes with vimentin suggests that it is incorporated into IFs. This finding implies that the MPR is not essential for NF incorporation into the IF network in this system. Previous studies by others have shown that digestion of the highly phosphorylated sidearm from assembled NFs had no effect on the integrity of the IF backbone (Chin et al., 1983). Our study confirms and extends these observations by directly demonstrating that the MPR is not essential for initiating the assembly of NF-M into stable IFs. Recent studies showing that extensive dephosphorylation of NFs *in vitro* by alkaline phosphatase had no effect on filament morphology (Carden et al., 1985; Hisanaga and Hirokawa, 1989) are further evidence supporting the lack of the MPR's role in assembly. Although the carboxy terminal regions of type III IF proteins have been shown to be unnecessary for assembly into filaments (Albers and Fuchs, 1987, 1989; van den Heuvel et al., 1987), many differences exist between the type III and type IV IFs (Steinert and Roop, 1988). One such difference is the presence of extended sidearm domains with extensive phosphorylation sites in NF-M and NF-H from all species but not in any other IFs (Steinert and Roop, 1988). However, the ability of MPR(-) NF-M to assemble into IFs suggests that, like type III IFs, the carboxy terminal extensions of type IV IFs may also be unnecessary for filament formation. These results validate

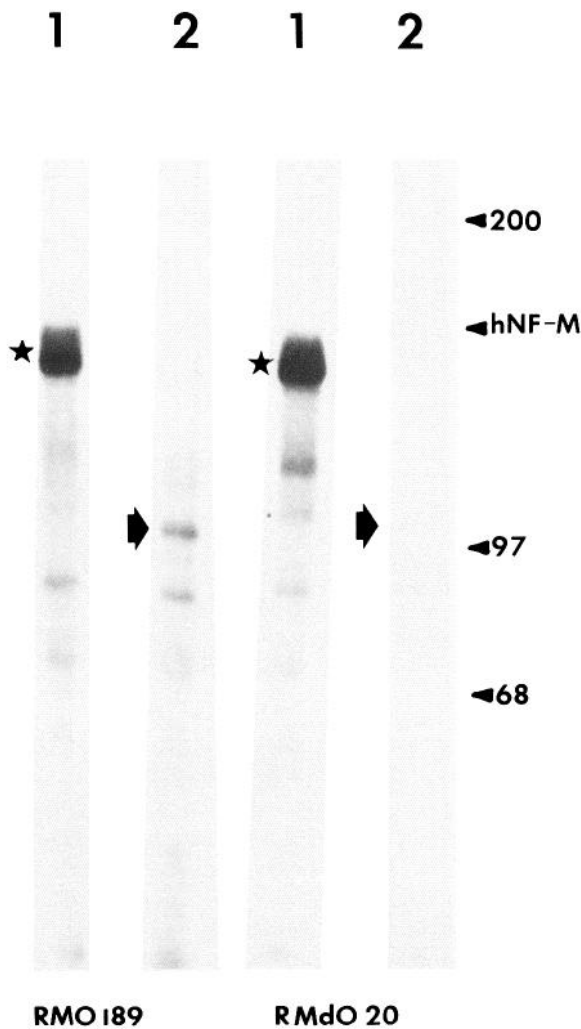


Figure 7. Both full-length NF-M and MPR(-) NF-M are phosphorylated in transfected L cells. *Lanes 1*, Extracts from MNA cells labeled with [32 P]PO $_4$. *Lanes 2*, Extracts from MNA-B cells labeled with [32 P]PO $_4$. The position of full-length NF-M from the L cells is indicated by stars and that of MPR(-) NF-M by arrows. The left panel was immunoprecipitated by RMO 189, an anticore NF-M mAb, and the right panel by RMdO 20, a mAb whose epitope is within the MPR. Note that RMdO 20 does not precipitate any MPR(-) NF-M. The electrophoretic mobility of NF-M isolated from human spinal cord along with M_r markers is shown at the right. The gel was exposed to film for 1 week at -70°C .

the pursuit of further studies of the residual sites of MPR(-) NF-M phosphorylation (not detectable when the MPR is present due to the extensive phosphorylation within the MPR) which may play a direct role in NF stability.

Although MPR(-) NF-M in transfected L cells lacks the major phosphorylation sites in human NF-M, it is phosphorylated at a low level in L cells. The site(s) phosphorylated in MPR(-) NF-M is presently unknown and may serve functions analogous to those attributed to phosphorylation sites in other IF proteins. For example, vimentin is phosphorylated within the amino terminal region, and repeated cycles of phosphorylation and dephosphorylation at this site(s) have been implicated in the process of repeated assembly-disassembly during the cell cycle (Inagaki et al., 1987; Chou et al., 1989; Evans, 1989). Nuclear lamins likewise are cyclically phosphorylated and dephosphor-

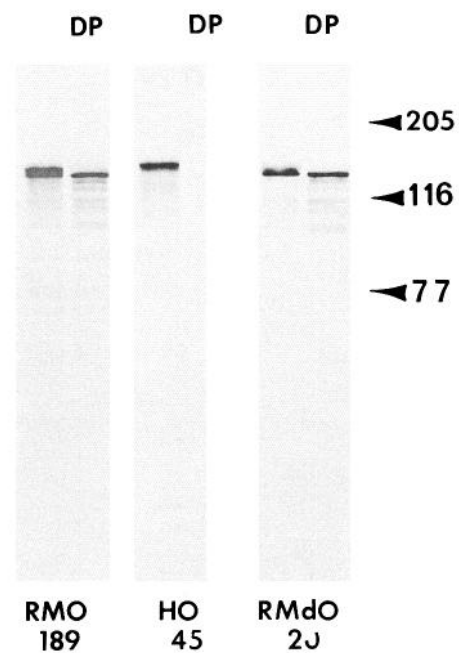


Figure 8. Dephosphorylation of full-length NF-M. Shown are gel replicas loaded with 30 μg of Triton-insoluble extracts which were either dephosphorylated (*lanes marked DP*) or not. These replicas were exposed to 3 mAbs of different phosphorylation-dependent specificities. RMO 189 is an anticore NF-M mAb whose reactivity is independent of the phosphorylation state of the molecule. HO 45 is an anti-MPR mAb whose reactivity is dependent on the presence of extensive phosphorylation of serines within the MPR (see Fig. 1). RMdO 20 is an anti-MPR mAb whose reactivity is dependent on the presence of several nonphosphorylated repeats of KSPV-based repeat. M_r markers are shown in kDa.

ylated in relation to the assembly and disassembly of the nuclear membrane during mitosis (Gerace and Blobel, 1980; Miake-Lye and Kirschner, 1985). Since the sequence KSPV has been shown to be the predominant phosphate acceptor motif in NF proteins (Lee et al., 1988a, b), the KSPV (aa 510–513) closer to the core region and outside the MPR (Myers et al., 1987) should be considered a potential phosphorylation site in human NF-M. Attempts were made to determine whether this KSPV is indeed the site of phosphorylation in MPR(-) NF-M. MAb specific for the nonphosphorylated form of the tetrapeptide sequence KSPV (Lee et al., 1988a, b) do not recognize the MPR(-) NF-M. These mAbs still do not recognize MPR(-) NF-M following dephosphorylation, suggesting that if this KSPV is phosphorylated, it is resistant to dephosphorylation. However, data from another laboratory indicate that a KSPV is present in the same position in rat NF-M (Napolitano et al., 1987) and that this KSPV is a site of phosphorylation (Xu et al., 1989). Other potential phosphorylation sites, including those present within the amino terminal portion of NF-M, may also be likely candidates (Sihag and Nixon, 1989). Many more definitive experiments, including 2-dimensional peptide mapping and sequencing, will be necessary to determine whether this KSPV in human NF-M and/or other serine residues are the authentic phosphate acceptor site(s) in MPR(-) NF-M.

The functional significance of NF phosphorylation in overall NF biology is unknown. Nevertheless, the resolution of phosphorylation site(s) outside of the MPR confirms the existence of at least 2 types of such sites the presence of which we described

in lamprey NF (Pleasure et al., 1989). In the lamprey, 2 distinct types of phosphorylation sites with different anatomical localizations were demonstrated. The first type was detected by MPR-specific mAbs, whereas the second was recognized by phosphorylation-dependent non-MPR mAbs. We have referred to the non-MPR site(s) of phosphorylation as "structural" sites since they were localized to axons of all sizes and may assume roles in NF assembly or filament maintenance that were indispensable to NF integrity. The MPR sites of phosphorylation in lamprey are occupied extensively only in large-diameter axons and may be involved in controlling axonal diameter (Pleasure et al., 1989). Our L-cell transfection system has not only allowed the resolution of both MPR and non-MPR phosphorylation sites, but will also provide an expression system to examine the regulation of phosphorylation of both these sites in human NF-M.

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