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MICROTUBULE-ASSOCIATED PROTEIN 2 (MAP2) EXPRESSION IN TRANSIENTLY AND STABLY TRANSFECTED P19 EMBRYONAL CARCINOMA CELLS

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To my husband Brent,
with love.

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ABBREVIATIONS

ANCOVA	:	analysis of covariance
BES	:	N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid
ddH_2O	:	distilled, deionized water
DMSO	:	dimethyl sulfoxide
EC	:	embryonal carcinoma
ECL	:	enhanced chemiluminescence
EDTA	:	ethylenediamine N,N,N′,N′-tetraacetic acid
EGTA	:	ethylene glycol bis (β-aminoethylether) N,N,N′,N′-tetraacetic acid
ELISA	:	enzyme-linked immunosorbent assay
GFAP	:	glial fibrillary acidic protein
HI-FCS	:	heat-inactivated fetal calf serum
HMW	:	high molecular weight
LB	:	Luria broth
LMW	:	low molecular weight
MAP	:	microtubule-associated protein
MEM	:	minimal essential medium
NTT	:	non-tyrosinatable tubulin
MT	:	microtubule
PEFA	:	p-aminoethylbenzene-sulfonyl fluoride
PGK	:	phosphoglycerate kinase
PBS	:	phosphate-buffered saline
PIPES	:	piperazine N,N-bis (2-ethane sulfonic acid)
RA	:	retinoic acid
RT	:	room temperature
SDS-PAGE	:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S-HRP	:	streptavidin-horseradish peroxidase
TAE	:	tris/acetate/EDTA
TE	:	tris/EDTA
WC	:	whole cell

ABSTRACT

In developing neurons, microtubule-associated protein 2 (MAP2) occurs as high molecular weight MAP2 (HMW-MAP2) and low molecular weight MAP2 (MAP2c) isoforms. MAP2 is developmentally regulated with MAP2c being expressed at high levels in immature, growing neurons, while HMW-MAP2 expression is restricted mainly to mature, stable neurons. This developmentally-regulated exchange in MAP2 expression correlates temporally with increased microtubule (MT) stability in differentiating neurons. Though both HMW-MAP2 and MAP2c have been shown to stabilize MTs *in vitro* and *in vivo*, their developmental regulation has suggested that HMW-MAP2 may stabilize MTs to a greater extent than MAP2c. To test this hypothesis, MAP2c and HMW-MAP2 were independently transfected into undifferentiated P19 embryonal carcinoma (EC) cells using the constitutive phosphoglycerate kinase (PGK) promoter to drive expression. MT stability was assayed by treating transfected cells with the MT depolymerizing drug colchicine for various lengths of time and measuring the number of transfected cells possessing stable MT bundles. MAP2c and HMW-MAP2 stabilized MTs to similar extents suggesting that, in developing neurons, the increase in MT stability can not be attributed directly to the developmental exchange in MAP2c and HMW-MAP2 expression.

Both MAP2c and HMW-MAP2 have been shown to promote the *in vitro* and *in vivo* assembly of MTs and inhibition of MAP2 expression in cultured neurons affects neuronal morphogenesis as indicated by a reduction in MT mass accumulation and neuritic growth. It is hypothesized that MAP2c and HMW-MAP2 are both required for neuronal morphogenesis. As a first step in testing this hypothesis, the developmentally-regulated, neuronal Tα1 α-tubulin promoter was used to drive overexpression of myc-tagged MAP2c (MAP2cmyc) in stably-transfected, neuronally-induced P19 cells. Comparison of relative MAP2 protein levels from untransfected and stable, MAP2cmyc-transfected neuronal cultures by Western blotting and ELISA showed higher MAP2c expression in the transfected cell line. Immunofluorescence microscopy was used to test the specificity and developmental timing of the Tα1 promoter. MAP2cmyc expression was restricted to neurons and was first detected three days after neuronal induction at the time of neuronal outgrowth. Protein sorting and neuronal morphology of MAP2cmyc-expressing neurons were also examined by immunofluorescence microscopy. In many neurons, MAP2cmyc was localized to all neurites and the morphology of these neurons appeared indistinguishable from untransfected, control neurons. These results illustrate the usefulness of the Tα1 promoter for targetting transgene expression specifically to neurons at the time of neurite outgrowth. Tα1-driven overexpression of MAP2c in P19 neurons did not appear to alter neuronal morphogenesis suggesting that other proteins may play more important roles in regulating this process.

RESUME

Chez les neurones en cours de développement, microtubule-associated protein 2 (MAP2) se retrouve sous forme de deux isoformes l'une dite de haut poids moléculaire (HMW-MAP2) et l'autre dite de faible poids moléculaire (MAP2c). L'expression de MAP2 est réglée en fonction des différents stades du développement neuronal; MAP2c étant fortement exprimée chez les neurones immatures en phase de croissance, alors que l'expression de HMW-MAP2 se produit principalement chez les neurones matures et stables. Ce changement développemental de l'expression de MAP2 coincide chronologiquement avec une augmentation de la stabilité des microtubules (MT) chez les neurones en cours de différenciation. Bien que HMW-MAP2 et MAP2c ont toutes les deux démontré un effet stabilisateur des MTs *in vivo* et *in vitro*, leur régulation développementale suggère que HMW-MAP2 pourrait avoir un effet stabilisateur plus important que MAP2c. Pour vérifier cette hypothèse, MAP2c et HMW-MAP2 ont été transfectées séparément dans des cellules de carcinome embryonnaire P19 non-différenciées en utilisant le promoteur constitutif de la phosphoglycerate kinase (PGK) pour la commande de l'expression. La stabilité des MTs fut mesurée en traitant des cellules transfectées avec un médicament ayant un effet dépolymérisateur des MTs, la colchicine, pendant des périodes de temps variables et en mesurant le nombre de cellules transfectées possédant des MTs groupés stables. MAP2c et HMW-MAP2 ont stabilisé les MTs à

un même degré ce qui suggère que chez les neurones en développement, l'augmentation de la stabilité des MTs ne peut être attribuée directement au changement développemental de l'expression de MAP2c et HMW-MAP2.

MAP2c et HMW-MAP2 ont toutes deux montré *in vivo* et *in vitro* un effet promoteur de l'assemblage des MTs et de plus, l'inhibition de l'expression de MAP2 chez les neurones en culture affecte la morphologie neuronale tel que démontré par une diminution de l'accumulation en masse de MTs et une réduction de la croissance des neurites. Il a été suggéré que toutes deux, MAP2c et HMW-MAP2 sont nécessaires à la morphogénèse neuronale. Comme première étape pour la vérification de cette hypothèse, le promoteur neuronal Tα1 α-tubuline (réglé selon le développement) a été utilisé pour contrôler la surexpression de MAP2c associée à myc (MAP2cmyc) dans des cellules P19 induites pour la croisance neuronale, transfectées stables. La comparaison par transfert Western et ELISA des niveaux relatifs de protéine MAP2 chez les cellules non-transfectées et stables avec ceux de cultures de cellules neuronales transfectées MAP2cmyc a démontré un niveau d'expression plus élévé chez les lignées cellulaires transfectées. La spécificité et la chronologie de l'activité du promoteur Tα1 au cours du developpement ont été évaluées par microscopie à immunofluorescence. L'expression de MAP2cmyc était limitée aux neurones et fut initialement détectée trois jours après l'induction neuronale, au moment de l'excroissance neuronale. La localisation des protéines et la morphologie des neurones exprimant MAP2cmyc furent également étudiées par microscopie à immunofluorescence. Chez plusieurs neurones, MAP2cmyc était localisée dans tous les neurites et leur morphologie semblait identique à celle des neurones témoins non-transfectés. Ces résultats illustrent l'utilité du promoteur Tα1 pour le ciblage de l'expression transgénique spécifique aux neurones rendus au stade de l'excroissance du neurite. La surexpression de MAP2c par le promoteur Tα1 chez les neurones P19 n'a pas semblé affecter la morphogénèse neuronale ce qui suggère que d'autres protéines pourraient jouer des rôles plus importants dans la régulation de ce processus.

INTRODUCTION

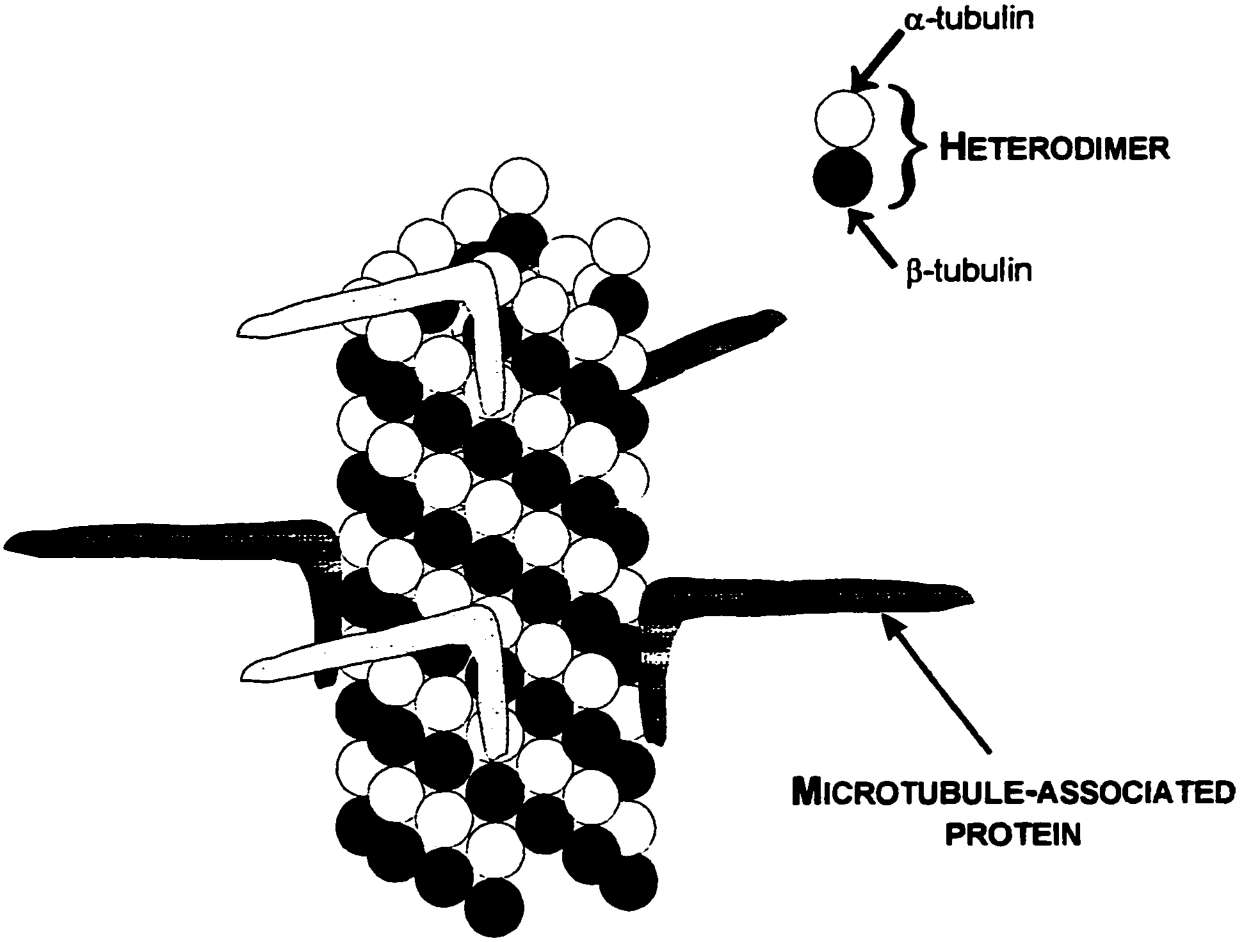
AN OVERVIEW OF MICROTUBULES

The eukaryotic cytoskeleton consists of three fibrillar components: microtubules, intermediate filaments, and microfilaments. These cytoskeletal fibres perform many cellular functions which include structural support, morphogenesis, and motility. Each fiber is composed of protein subunits which allows for the highly dynamic nature of the cytoskeletal network. These protein subunits polymerize into fibers which can later depolymerize, allowing for the rapid reorganization of cytoskeletal components required for many cellular activities and morphogenic events.

Microtubules (MTs), with a diameter of 25 nm, are the largest of the cytoskeletal fibrils. They are the constituents of many cellular structures such as the microtubule bundles in neuritic processes, axonemes of flagella and cilia, spindles of mitotic cells, and radial MT arrays of interphase cells. These MT structures perform a variety of functions which include morphogenesis, cell motility, chromosome segregation, and intracellular transport.

MTs are composed primarily of the protein tubulin which polymerizes to form the walls of microtubules (Fig. 1). Tubulin is a 100 kD heterodimer composed of two polypeptides designated α-tubulin and ß-tubulin. Tubulin heterodimers polymerize end to end to form protofilaments. In the cells of most eukaryotes,

Figure 1. Microtubular structure with associated MAP proteins.


α-tubulin
HETERODIMER
β-tubulin
MICROTUBULE-ASSOCIATED PROTEIN

thirteen protofilaments interact laterally to generate a microtubule (Bershadsky and Vasiliev, 1988). A variety of α-tubulin and ß-tubulin proteins are expressed by most eukaryotic species. Some of this diversity is attributed to expression of distinct but related proteins encoded by highly conserved multigene families (reviewed by Joshi and Cleveland, 1990; Murphy, 1991; Ludueña *et al.*, 1992; Ludueña, 1993). The protein product of a single tubulin gene is referred to as an isotype. Posttranslational modifications of tubulin polypeptides account for the remaining variability and produce what are defined as tubulin isoforms. These modifications include carboxy-terminal detyrosination/tyrosination and acetylation of α-tubulin, phosphorylation of ß-tubulin, and polyglutamylation of both α- and ß-tubulin (Kumar and Flavin, 1981; L'Hernault and Rosenbaum, 1985; Gard and Kirschner, 1985; Eddé *et al.*, 1990). Also, the carboxy-terminal glutamyl-tyrosine dipeptide of α-tubulin can be removed to generate a non-tyrosinatable tubulin (NTT) isoform (Paturle-Lafenechère *et al.*, 1991). Recently, the list of posttranslational modifications has grown to include polyglycylation (Redeker *et al.*, 1994; Rüdiger *et al.*, 1995).

Along with tubulin, members from a group of proteins called microtubule-associated proteins (MAPs) make up MTs *in vivo*. MAPs were first discovered due to their ability to copurify with tubulin through successive cycles of MT assembly and disassembly (Borisy *et al.*, 1975). They were originally classified based on their relative mobilities determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Murphy and Borisy, 1975; Sloboda *et al.*, 1976; Weingarten *et al.*, 1976). Early studies revealed their association with the outer surface of MTs (Fig. 1) and their *in vitro* MT assembly-promoting ability (Dentler *et al.*, 1975; Murphy and Borisy, 1975; Sloboda *et al.*, 1976).

MAPs are now classified into two categories based on cellular function. Motor MAPs, which include dyneins and kinesins, produce motile force by hydrolyzing ATP, thus enabling MT-dependent cytoplasmic transport (reviewed by Bloom, 1992; Scholey and Vale, 1994; Collins, 1994). Structural MAPs, which include MAP1a, MAP1b, MAP2, tau, and MAP4, are classified based on their ability to bind to MTs and stimulate MT assembly (reviewed by Chapin and Bulinski, 1992; Maccioni and Cambiazo, 1995).

Cloning and sequencing of these MAPs has revealed a further subdivision within the structural MAP family. Members of both subgroups possess MT-binding domains containing repeated sequence motifs that are unrelated (Noble *et al.*, 1989). MAP1a and MAP1b belong to the subgroup of heat-labile MAPs that possess a MT-binding domain located in the amino-terminal half of the protein. This binding domain consists of a motif, containing a similar core sequence (KKEX, where K is lysine, E is glutamic acid, and X is either a hydrophobic or acidic residue), repeated approximately 21 times for MAP1b and 11 times for MAP1a (reviewed by Chapin and Bulinski, 1992; Maccioni and Cambiazo, 1995)

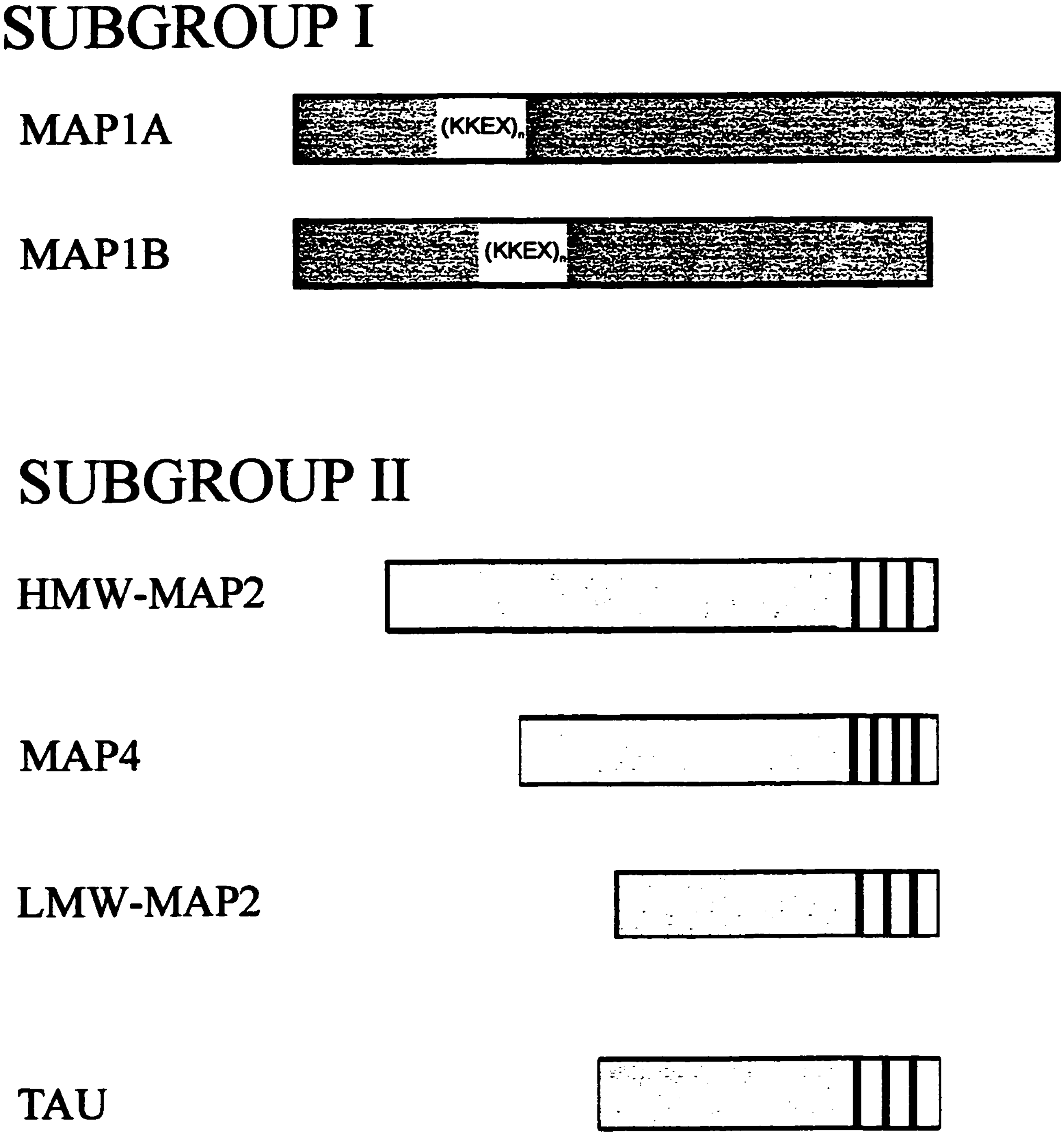
(Fig. 2). Regions flanking the repeats also contribute to MT binding. The heat-stable MAPs, which include MAP2, MAP4 and tau, make up the second structural MAP subgroup. The MT-binding domain in these MAPs is situated near the carboxy-terminus and consists of three or four imperfect repeats of a highly conserved 18 amino acid motif, separated by spacer regions of 13 or 14 amino acids (reviewed by Chapin and Bulinski, 1992) (Fig. 2).

Members of the structural MAP family demonstrate different tissue expression patterns, which may imply unique roles for MAP proteins in these tissues. MAP4 is expressed in a range of tissue types such as brain, liver, and kidney. MAP1 species are enriched in neuronal cells but are also found in other tissue and cell types. MAP2 and tau species are expressed predominantly in neural tissue (reviewed by Chapin and Bulinski, 1992). In fact, tau and MAP2 expression is restricted almost exclusively to neurons.

MICROTUBULE-ASSOCIATED PROTEIN 2

Of the structural neuronal MAPs, MAP2 is the most abundant, brain-specific MAP. In rat brain, it exists in two high molecular weight (HMW) forms, MAP2a and MAP2b, and two low molecular weight (LMW) forms, MAP2c and MAP2d (Binder *et al.*, 1984; Burgoyne and Cumming, 1984; Garner *et al.*, 1988; Doll *et al.*, 1993). However, in mouse brain, only one HMW form is resolved by SDS-PAGE (Crandall and Fischer, 1989). MAP2 isoforms are encoded by a single copy gene assigned to human chromosome

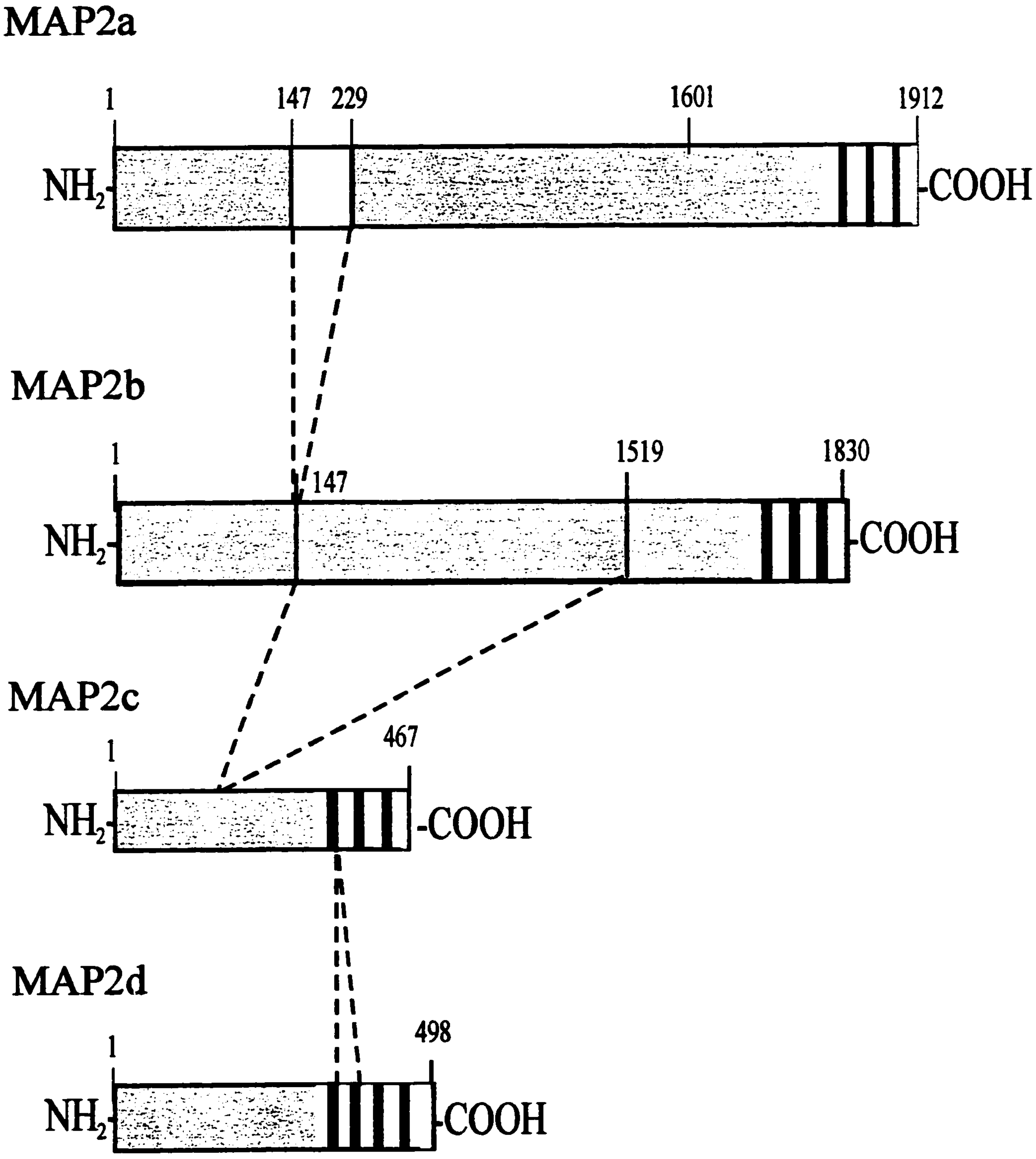
Figure 2. Diagrammatic comparison of the primary structure of structural MAP proteins. Subgroup I consists of the heat labile MAPs, MAP1a and MAP1b which have a MT-binding domain (light grey box) located in the amino-terminal portion of the protein, containing the repeated core sequence $(KKEX)_n$, where n=11 for MAP1a and n=21 for MAP1b. HMW-MAP2, MAP4, LMW-MAP2, and tau make up the heat stable MAP subgroup II. These proteins have a carboxy-terminal MT-binding domain (light grey box) that consists of three or four imperfect repeats (black boxes) of a highly conserved 18 amino acid motif separated by spacer regions.


SUBGROUP I
MAP1A
(KKEX)n
MAP1B
(KKEX)n
SUBGROUP II
HMW-MAP2
MAP4
LMW-MAP2
TAU

2q34-35 (Neve *et al.*, 1986; Garner and Matus, 1988). Alternative splicing of the primary transcript is responsible for production of the various MAP2 isoforms. The HMW-MAP2 isoforms are encoded by a 9 kb mRNA. MAP2a mRNA contains an additional 246 nucleotides near the 5′ terminus of the 9 kb mRNA, resulting in a protein 82 amino acids larger than MAP2b (Chung *et al.*, 1996) (refer to Fig. 3). By SDS-PAGE, the relative molecular masses of the MAP2a and MAP2b polypeptides are 288 and 280 kDa, respectively. The LMW-MAP2 species are derived from a 6 kb mRNA, generated by removal of the internal region of the primary transcript. This internal region encodes the 1372 amino acids of the HMW-MAP2 projection arm domain (Garner and Matus, 1988). MAP2c and MAP2d have apparent molecular masses of 69 and 74 kDa and contain 467 and 498 amino acids, respectively (Doll *et al.*, 1993; Olesen, 1994). The difference in length of 31 amino acids is due to an additional, fourth repeat in the MT-binding domain of MAP2d (Doll *et al.*, 1993) (see Fig. 3).

In addition to tissue-specific expression, MAP2 is developmentally regulated. MAP2d is expressed in glial cells and appears later in development, coincident with the later differentiation of glial cells relative to neurons (Doll *et al.*, 1993). MAP2b is present throughout development and shows little variation in relative abundance. MAP2c is expressed at its highest level early in neuronal development until postnatal day 10 and is, therefore, referred to frequently as embryonic or juvenile MAP2. In rat brain, MAP2c levels decrease rapidly

Figure 3. Diagramatic comparison of MAP2 isoforms. The two HMW-MAP2 proteins both possess a MT-binding domain (light grey box) with three basic repeats (black boxes) but MAP2a contains an extra 82 amino acid sequence near the amino terminus. The LMW-MAP2 proteins are derived from the alternative splicing of the primary transcript which removes an internal 1372 amino acids (dotted lines) found in MAP2b. MAP2d possesses an extra repeat in the MT-binding domain making it 31 amino acids larger than MAP2c.


MAP2a
1
147
229
1601
1912
NH2-
-COOH
MAP2b
1
147
1519
1830
NH2-
-COOH
MAP2c
1
467
NH2-
-COOH
MAP2d
1
498
NH2-
-COOH

between postnatal days 10 and 20, which corresponds to the period of synaptogenesis when neuronal networks become consolidated (Riederer and Matus, 1985). MAP2a is not expressed in newborn rat brains but begins to accumulate between postnatal days 10 and 20, reaching its peak in the adult brain (Binder *et al.*, 1984; Burgoyne and Cumming, 1984). This developmentally regulated exchange in MAP2c and MAP2a expression temporally coincides with the increased stabilization of MTs in developing neurons. Specifically, MAP2c expression is associated with growing neurons, when MT plasticity is greatest, while MAP2a expression and increased MT stability of "adult" neurons are correlated temporally. The association of MAP2c and neuronal growth is supported by its continued expression in tissues where neuronal growth continues even during adult life such as the mammalian olfactory bulbs (Viereck *et al.*, 1989) and the mammalian retina (Tucker and Matus, 1988). These findings suggest that MAP2c may play a role in the initial morphogenesis of developing neurons and HMW-MAP2 isoforms, like MAP2a, may function specifically in stabilizing the neuronal MT cytoskeleton.

The availability of MAP2 cDNA clones (Wang *et al.*, 1988; Kindler *et al.*, 1990; Doll *et al.*, 1990) has allowed the investigation of MAP2 function in non-neuronal cells, where MAP2 is not normally expressed. Various properties of both HMW-MAP2 and MAP2c have been assayed by transiently transfecting non-neuronal cells with the cDNAs for each protein. Lewis *et al.* (1989) performed the first set of transfection experiments with

HMW-MAP2 and showed not only its binding to non-neuronal MTs but also its ability to bundle them. Later studies demonstrated, after deleting most of the amino-terminal arm domain, that only a short sequence near the carboxy-terminus of HMW-MAP2 is essential for MT bundling (Lewis and Cowan, 1990). Subsequent investigations with MAP2c, MAP2d, and the related protein tau also show that overexpression of these proteins induces the reorganization of interphase MTs (Kanai *et al.*, 1989; Knops *et al.*, 1991; Takemura *et al.*, 1992; Weisshaar *et al.*, 1992; Umeyama *et al.*, 1993). In particular, high levels of expression result in the formation of MT bundles, resembling the bundled MTs found in neuronal processes. However, transgenic mice, expressing MAP2c in the adult brain, show no detectable alteration in the morphology or arrangement of neurons, suggesting that MAP2c is required for neuromorphogenesis but is not directly involved in its regulation (Marsden *et al.*, 1996). MAP2 antisense studies, on the other hand, show that reducing MAP2 expression hampers MT stability maintenance and neurite elongation in cultured cortical neurons (Sharma *et al.*, 1994) and inhibits neurite outgrowth in neuronally-induced P19 cells (Dinsmore and Solomon, 1991) and cultured cerebellar macroneurons (Caceres *et al.*, 1992).

Besides altering their organization within the cytoplasm, MTs are stabilized against MT depolymerizing agents when genes for HMW-MAP2 (Lewis *et al.*, 1989), MAP2c (Takemura *et al.*, 1992; Ferhat *et al.*, 1996), MAP2d (Ferhat *et al.*, 1996), and the related MAP, tau, (Takemura *et al.*, 1992; Baas *et al.*, 1994) are transfected into non-neuronal cells. Takemura *et al.* (1992) compared the MT stabilizing abilities of MAP2c and tau in transiently transfected non-neuronal cells. By measuring the percentage of transfected cells containing nocodazole-resistant MT bundles after 20 minute and two hour exposures, the investigators concluded that MAP2c-bundled MTs were more stable than MTs bundled by tau. A similar comparison between the extent of MT stabilization conferred by HMW-MAP2 and MAP2c has yet to be attempted.

MAP2 isoforms exhibit a polarized distribution in neurons. Immunohistochemical studies show MAP2 isoforms to be restricted to dendrites in most regions of the developing brain (Bernhardt and Matus, 1984; Burgoyne and Cumming, 1984). MAP2c protein demonstrates sorting to dendrites in the adult brain of transgenic mice (Marsden *et al.*, 1996). However, unlike HMW-MAP2 isoforms, MAP2c has also been localized to the axons of developing motor neurons and retinal ganglion cells (Tucker *et al.*, 1988; Albala *et al.*, 1995; Tucker and Matus, 1988). Also, Meichsner *et al.* (1993) demonstrated labelling of myc-tagged-MAP2c in all processes of transfected hippocampal neurons grown in culture. Similar studies on primary cultured spinal cord neurons show MAP2c entering the proximal region of axons (Kanai and Hirokawa, 1995). To date, the effect of MAP2c on neuronal morphogenesis and its subcellular sorting in developing neurons remains unclear.

Transient transfections of cloned genes into cultured cells has provided clues into the roles particular proteins play in a cellular environment. For example, transfection studies with MAP2 and tau have elucidated some of the functional properties of these proteins in a non-neuronal environment. However, transient transfection studies have several inherent limitations. First, transfection efficiencies can vary greatly between individual experiments, making comparative studies difficult. Second, transfection efficiencies for some cultured cell types can be very low, providing very little material for study. Cultured neurons, for example, typically show transfection efficiencies below five percent (Kanai and Hirokawa, 1995) and sometimes can be as low as one in a thousand (Meichsner *et al.*, 1993). Third, there is a variation in the plasmid copy number picked up and expressed by individual transfected cells. The amount of exogenous protein produced by each cell will therefore be different and, hence, the biological effect observed may also be variable among the population of transfected cells. Finally, many of these transient transfection studies are conducted using cell types that do not normally express the protein of interest. Ideally, protein function studies should be conducted using a cell type where the studied protein naturally occurs, ensuring that physiological requirements are met and proteins normally co-expressed and potentially affecting its behaviour are present.

P19 EMBRYONAL CARCINOMA CELLS

Embryonal carcinoma (EC) cell lines offer an alternative to transfection studies. EC cell lines are developmentally multipotent, and usually, when cultured at high density (Nicolas *et al.*, 1975; McBurney, 1976) or aggregated (Martin and Evans, 1975), will spontaneously differentiate into a wide variety of cell types. The mouse P19 EC cell line (McBurney and Rogers, 1982) differentiates inefficiently under these conditions but can be induced to differentiate along a limited number of pathways upon exposure to non-toxic concentrations of specific drugs (McBurney, 1993). P19 differentiation is also relatively synchronous with reproducible kinetics and cell type characteristics (MacPherson and McBurney, 1995). Dimethyl sulfoxide (DMSO) induces P19 cell aggregates to differentiate into derivatives of mesoderm and endoderm (McBurney *et al.*, 1982). On the other hand, retinoic acid (RA) induces P19 cells to differentiate into derivatives of neuroectoderm, composed mainly of neurons and glia (Jones-Villeneuve *et al.*, 1982). Because P19 cells are easily induced along these specific differentiation pathways, they are valuable for studies of muscle and neuronal development (reviewed in McBurney, 1993; Bain *et al.*, 1994).

The P19 cell line has been used to investigate factors infuencing the stability of MTs during neuronal morphogenesis (Falconer *et al.*, 1989; Falconer *et al.*, 1992; Falconer *et al.*, 1994; Laferrière and Brown, 1996). Within 24 hours of RA-induction, P19 cells form a colchicine-stable bundle of acetylated MTs (Falconer *et al.*, 1989). MAP2 is absent from undifferentiated P19 cells, but is expressed following RA-induction at the time of neurite extension (Falconer *et al.*, 1989). As expected, MAP2c is the first MAP2 isoform expressed during neuromorphogenesis. By Western blotting, MAP2c is initially detected two days following neuronal induction, while HMW-MAP2 is detected six days after RA-induction (Falconer *et al.*, 1992). ELISA results demonstrate that MTs become increasingly more stable as neuronal differentiation proceeds (Laferrière and Brown, 1996). The developmental regulation of MAP2 expression in neuronally differentiating P19 cells is correlated temporally with increasing MT stability (Falconer et *al.*, 1992) which reflects what is seen in brain (reviewed by Matus, 1991). The P19 cell line appears to be a good model system for investigating the molecular mechanisms modulating MT stability in developing neurons.

P19 cells are also amenable to genetic manipulation, thus providing an appropriate system for investigating the effects of transgene expression on specific developmental processes (reviewed in McBurney, 1993; MacPherson and McBurney, 1995). For example, the phosphoglycerate kinase (PGK-1) constitutive promoter was used to direct expression of Hox 1.6 (Pratt *et al.*, 1993), MyoD (Skerjanc *et al.*, 1994) and ß-galactosidase (Morassutti *et al.*, 1994) in differentiating P19 cells. The PGK-1 promoter was also used to drive expression of a MAP2 antisense

RNA in differentiating P19 cells (Dinsmore and Solomon, 1991). However, although P19 cells are easily transfected, clones stably expressing transgenes have proven difficult to isolate (McBurney *et al.*, 1994). These cells are cotransformed inefficiently and transgenes are inactivated at high frequency resulting in their mosaic expression (McBurney *et al.*, 1994; Schmidt-Kastner *et al.*, 1996). Also, although the constitutive PGK-1 promoter shows transgene expression in all tissues of transgenic mice, the level of expression is not uniform in all cells (McBurney *et al.*, 1994).

DEVELOPMENTAL PROMOTERS

The use of developmentally regulated, tissue-specific promoters provides a means to analyze the effect of transgene expression in the specialized environment of a particular tissue type. For example, Rudnicki *et al.* (1988) have shown, using the human cardiac actin (C^H-actin) promoter, appropriate developmental and muscle-specific expression of human cardiac actin and herpes simplex virus thymidine kinase proteins in DMSO-treated P19 cells. Regions in the C^H-actin promoter required for efficient and appropriate expression were identified using deleted versions of the promoter to drive ß-galactosidase expression in stably transfected P19 cells (Pari *et al.*, 1991). This muscle-specific promoter was also used to transform P19 cells with the H-ras oncogene (Rudnicki *et al.*, 1989).

Since P19 cells are inducible along the neuronal pathway, a promoter capable of targetting gene expression to neurons would be useful in assessing the effects of specific proteins on neuronal differentiation. One such candidate to direct neuron-specific expression is the Tα1 α-tubulin promoter. In the developing rat embryo, the Tα1 α-tubulin gene is expressed specifically and at high levels in differentiating neurons. Tα1 α-tubulin mRNA levels remain high during the development of the nervous system, when the extent of neuronal growth is great, but diminish concomitant with target contact (Miller *et al.*, 1987). In mature motor neurons (Miller *et al.*, 1989) and sympathetic neurons (Mathew and Miller, 1990), Tα1 mRNA levels increase in response to axonal injury. Following axonal regeneration, Tα1 mRNA levels decrease in these neurons. Mature neurons from the central nervous system (CNS) respond similarly to axotomy except that regeneration does not occur and Tα1 mRNA levels remain high (Tetzlaff *et al.*, 1991). Exposure to growth factors from the neurotrophin family also results in elevating Tα1 expression in mature sympathetic neurons (Mathew and Miller, 1990). Tα1 α-tubulin expression is therefore associated with developmental stages, regeneration events, and growth cues requiring or inducing extensive neuronal growth.

The Tα1 α-tubulin gene was isolated and its promoter determined using 1.1 kb of 5′ flanking sequence to drive nuclear-localized β-galactosidase expression in transgenic mice (Gloster *et al.*, 1994). Transgenic β-galactosidase expression is observed only in neurons, is repressed following target contact, and is reinduced following axonal injury (reviewed by Miller *et al.*, 1996). In the mature nervous system, the expression pattern of the ß-galactosidase transgene is similar to that of the endogenous Tα1 mRNA (Bamji and Miller, 1996). In P19 cells, ß-galactosidase expression from the Tα1 promoter is restricted to neurons and shows proper regulation following exposure to RA (Rogers *et al.*, 1995). The Tα1 promoter appears to be an excellent candidate to drive neuronal-specific expression of a transgene in differentiating neurons.

THESIS OBJECTIVES

In differentiating neurons, MAP2c expression coincides with neuronal growth and greater MT plasticity while HMW-MAP2 expression is associated with the consolidation of the neuronal MT network. Transfection studies in non-neuronal cells demonstrate that both MAP2c and HMW-MAP2 stabilize MTs *in vivo*. Therefore, MAP2c and HMW-MAP2 may possess different MT stabilizing abilities required at specific stages of neuronal morphogenesis. The specific roles of MAP2c and HMW-MAP2 in neuronal morphogenesis remains unclear. MAP2 antisense studies show that reducing total MAP2 expression impedes both the elongation and maintenance of growing and existing neurites, respectively.

These observations have led to the suggestion that HMW-MAP2 may stabilize MTs to a greater extent than MAP2c and that both

MAP2 proteins are required for neuronal morphogenesis. The goal of the first part of this study was to compare the stability of MTs bound by MAP2c and HMW-MAP2 *in vivo*. The goal of the second part of this study was to examine the effects of MAP2c overexpression on neuronal morphogenesis.

MATERIALS AND METHODS

PLASMID CONSTRUCTS

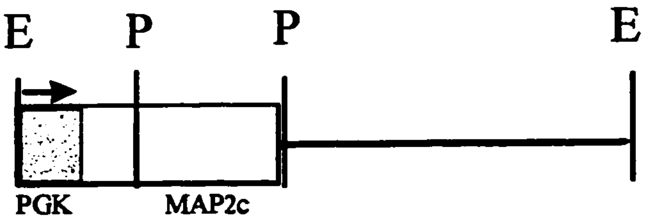
A 1.75 kb NotI fragment containing the MAP2c coding region (Kindler *et al.*, 1990) from pCMVneo2c (a kind gift from Dr. C.C. Garner, University of Alabama, Birmingham) was made blunt-ended using Klenow polymerase and subcloned into pPOP (generously provided by Dr. M.W. McBurney, University of Ottawa), previously digested with SmaI, generating the plasmid pPGK2c.

For the MAP2b expression vector, a new vector was derived from the pPOP plasmid by replacing the BamHI site with a NotI site, generating the plasmid pPOPnot. Briefly, the BamHI containing fragment of pPOP was removed by restriction digest using SacI and SalI. A linker, designed to introduce the NotI restriction site, was ligated into the digested plasmid. A 5.85 kb NotI fragment containing the MAP2b coding region (Kindler et *al.*, 1990) from pCMVneo2b (also generously provided by Dr. C.C. Garner) was subcloned into the NotI site of pPOPnot, generating the plasmid pPGK2b. A third construct consisted of a 1.75 kb myc-tagged MAP2c NotI fragment from pCMVneo2cmyc (also provided by Dr. C.C. Garner) subcloned by the same method into pPOPnot generating pPGK2cmyc. Both pPOP and pPOPnot plasmids contain the mouse phosphoglycerate kinase (PGK-1) promoter which was used to drive MAP2c, MAP2cmyc, and MAP2b expression in undifferentiated P19 EC cells.

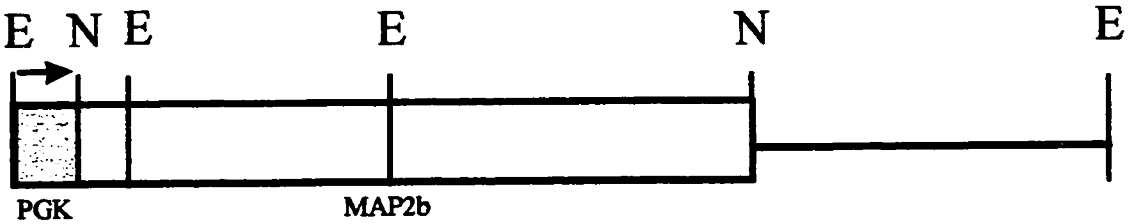
The myc-tagged MAP2c NotI fragment from pCMVneo2cmyc was also subcloned by blunt-end ligation into the XhoI multicloning site of p253 (a generous gift from Dr. F.D. Miller, Montreal Neurological Institute, McGill University) producing the plasmid pTα12cmyc. This plasmid contains 1.1 kb of 5′ flanking sequence from the Tα1 α-tubulin gene which includes the promoter and was used to drive expression of myc-tagged MAP2c in P19 cells. All plasmid identities were verified by restriction mapping (refer to Fig. 4 for plasmid maps). The plasmid pPGKpuro (obtained from Dr. M.W. McBurney) was cotransfected with pTα12cmyc to permit the selection of stably transfected clones in the presence of puromycin.

Ligations were performed overnight at 14°C in 15 μl volumes, using 2 units of T4 ligase, 2 μl of 5 mM ATP, and an excess of insert to vector DNA. The linker and all insert DNA sequences were phosphorylated with kinase prior to performing ligations. All restricted vectors were dephosphorylated using calf intestinal phosphatase prior to performing ligations. All kinase and phosphatase reactions were carried out as outlined in the manufacturer′s instructions. Restriction endonucleases, ligase, dNTPs, and ATP were purchased from Boehringer Mannheim (Laval, Que.). Polynucleotide kinase and Klenow DNA polymerase were obtained from New England Biolabs (Mississauga, Ont.). Alkaline phosphatase was purchased from Pharmacia (Baie d′Urfé, Que.).

Figure 4. Maps of plasmid expression vectors used for transient and stable transfections of P19 cells. All plasmids used for transient transfections contain the PGK-1 (phosphoglycerate kinase) promoter to drive transgene expression. The neuron-specific Tα1 α-tubulin promoter was used for the stable transfection of myc-tagged MAP2c. Restriction sites for EcoRI (E), PstI (P), and NotI (N) are shown.



pPGK2c (5.39 kb)



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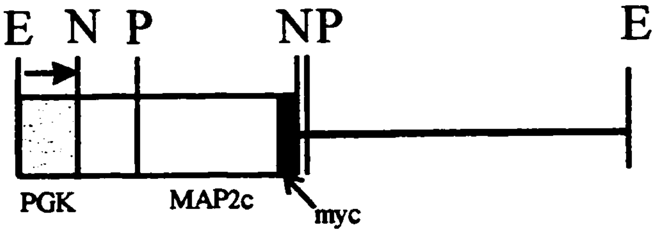
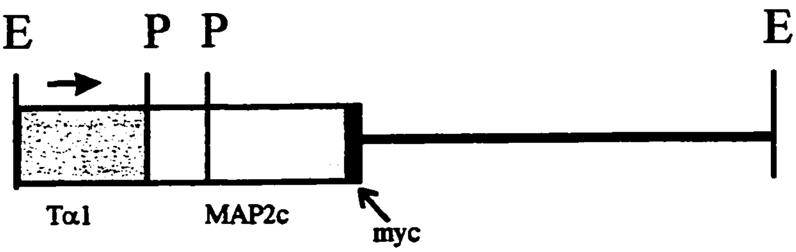


pPGK2cmyc (5.42 kb)



pTα12cmyc (6.49 kb)

PLASMID PREPARATION

Methods for bacterial transformation, plasmid DNA alkaline lysis minipreparation, and restriction enzyme digests were obtained from Sambrook *et al.* (1989). Restriction digests were performed in a reaction volume of 20 µl, using 1 µl of minipreparation plasmid DNA or 100-200 ng of large-scale plasmid DNA (to be described), 1 µl (10 units) of the appropriate endonuclease, and incubated for a minimum of 1 hr at the appropriate temperature. DNA fragments were separated on 0.8% agarose/Tris-acetate-EDTA (TAE) (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) gels, containing ethidium bromide, and photographed for analysis (Sambrook *et al.*, 1989).

To obtain large quantities of plasmid DNA for transfection, each plasmid was used to transform competent *E. coli* DH5α bacteria, which were then plated onto LB agar plates containing 100 µg/ml ampicillin, and grown overnight at 37°C. Colonies were aseptically transferred into 2 ml T-broth cultures, also containing 100 µg/ml ampicillin, and grown on a shaker overnight at 37°C. To verify the identity of a plasmid, 1 ml of the broth culture was used for plasmid DNA preparation and analyzed by restriction mapping. The remaining culture was used to inoculate 250 ml of T-broth, containing 100 µg/ml ampicillin, which was grown at 37°C, overnight with agitation. The bacteria were harvested by centrifugation and the plasmid DNA was isolated by the alkaline-lysis method (Birnboim and Doly, 1979). Plasmid DNA was resuspended in TE (10 mM Tris,1 mM EDTA, pH 8.0) or distilled, deionized water (ddH_2O), quantitated by UV absorbance spectrophotometric analysis at 260 nm, and identity verified by restriction mapping.

CELL CULTURE

The P19 EC cell line (McBurney and Rogers, 1982) was maintained at 37°C and 5% CO_2 in α-MEM (Gibco BRL, Burlington, Ont.), supplemented with 10% heat-inactivated fetal calf serum (HI-FCS)(Gibco BRL) and 1% of a 100X antibiotic/antimycotic stock (penicillin, streptmycin and fungizone)(Gibco BRL). Cells were passaged every 2 days using 0.25% trypsin, 1 mM EDTA in calcium- and magnesium-free phosphate-buffered saline (PBS). For transient transfection assays, cells were seeded on 18 mm round coverslips (cs) at $2x10^4$ cells per cs in 12-well tissue culture plates. For RA-induced neuronal differentiation, cells were seeded at different densities depending on the processing day. Undifferentiated cells, and cells processed 2, 3, and 4 days after RA addition were seeded on 22x22 mm cs at $4x10^4$ cells per cs in 6-well tissue culture dishes. The cell density for samples processed 6 and 8 days after RA addition was $2x10^4$ and for days 10 and 12 was $1x10^4$ cells per cs. For differentiation in 100 mm dishes, seeding densities of $4x10^5$ cells per dish for undifferentiated cells, day 2, 3, and 4 cells and $2x10^5$ cells per dish for all later days were used. All initial seeding was done using cells growing in α-MEM, supplemented with 10% HI-FCS and containing antibiotics. Twenty-four hours following initial plating, the medium was replaced with α-MEM, 10% HI-FCS, antibiotics and 10^{-6} M RA to induce neuronal differentiation. This medium was replaced 24 hours later with a serum-free medium lacking RA to inhibit cell proliferation and produce a culture enriched for neurons (MacPherson and McBurney, 1995). The differentiating cells were maintained in this medium which was changed every 2 days. RA stocks were made up at 10^{-3} M in ethanol, stored at 4°C and diluted to 10^{-6} M with medium prior to use.

For MT depolymerization experiments, approximately 24 hr following transfection, fresh, prewarmed growth medium containing 1 μg/ml colchicine was added to the cells for specified exposure times before processing for microscopy. Colchicine was kept as a filter-sterilized, 1 mg/ml stock in ddH_2O and stored at -20°C. The transfected cells were exposed to colchicine for 2, 3, or 4 hr. The percentage of transfected cells containing stable MT bundles was determined for 0, 2, 3, and 4 hr exposure times. In most cases, a minimum of 100 transfected cells was counted for each experiment. To account for variability between individual experiments, three separate experiments were conducted for each MAP2 isoform. Experiments were performed on different days and included all colchicine exposure times. HMW-MAP2 transfection experiments yield lower transfection efficiencies than those conducted with MAP2c. Therefore, percentages of stable MT bundles remaining after each colchicine exposure time were plotted and the slopes generated were used to compare the MT stabilizing abilities of HMW-MAP2 and MAP2c. An analysis of covariance (ANCOVA) was performed to determine if the slopes for MAP2c and HMW-MAP2 were significantly different. An alpha of 0.05 was used and all the assumptions of the test were met.

DNA TRANSFECTIONS

P19 cells, grown overnight on cs in α-MEM, supplemented with 10% HI-FCS and containing antibiotics, were transiently transfected by the calcium phosphate DNA precipitation method as described by Chen and Okayama (1987), with slight modifications. The calcium-phosphate-DNA mixture was prepared by adding 50 μl of 2.5 M $CaCl_2$, 40 μg of DNA, and ddH_2O to bring the total volume to 500 μl. After briefly vortexing the solution, 500 μl of 2X BES (N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid) -buffered saline (pH 6.86) was added and allowed to sit for 15 min at room temperature (RT). After gentle mixing, 200 μl (8 μg of DNA) of the final solution was added slowly to each cs and incubated at 37°C for 8 hours. The precipitate was then removed with 2 washes of PBS, then 1 ml fresh α-MEM, containing 10% HI-FCS and antibiotics, was added. The transfected cells were fixed and stained the following day. To obtain stably transfected clones expressing myc-tagged MAP2c, P19 EC cells were transfected by the same method with 13 μg of pTα12cmyc and 2μg of pPGKpuro and incubated for 8 hours. The cells were treated as described above but were trypsinized and seeded onto 100 mm dishes at $1x10^6$ cells per dish 24 hours after transfection. The following day, the medium was replaced with a selective medium composed of α-MEM, 10% HI-FCS, antibiotics, 7 x 10^{-6} % ß-mercaptoethanol, and 2 μg/ml puromycin. This medium was replaced every 2 days for 6 days until discrete colonies were visible. Puromycin stocks were made up at 2 mg/ml in PBS, filter-sterilized, stored at 4°C, and diluted 1:1000 in medium prior to use. ß-mercaptoethanol stocks were filtered-sterilized, stored at 4°C, and were made by adding 7 μl of ß-mercaptoethanol to 10 ml of PBS, which was diluted 1:100 in medium prior to use. Colonies were selected under a dissecting microscope using a pipetman equipped with a yellow tip. Individual colonies were lifted off the culture dish surface, rinsed in PBS drops, resuspended in drops of trypsin/EDTA and transferred to 1 ml of fresh selective medium in 12-well plates. These clones were later expanded in 6-well plates in the presence of puromycin. To screen for expression of the MAP2cmyc transgene, each clone was induced to differentiate by RA on coverslips and was stained with an antibody that recognizes the human c-myc tag.

IMMUNOFLUORESCENCE MICROSCOPY

Cells grown on cs were briefly rinsed in calcium- and magnesium-free PBS and fixed by one of three methods. 1) Cells were simultaneously fixed and extracted in 3.7% formaldehyde (v/v), 0.25% glutaraldehyde (v/v), and 0.5% Triton X-100 (v/v) in PEM buffer (80 mM PIPES, 5 mM EGTA and 1 mM $MgCl_2$, pH 6.9) for 10 min. 2) Cells were fixed and post-extracted as described by

Stefanini *et al.* (1967) in 4% paraformaldehyde (w/v) and 14% picric acid (v/v) in 0.25 M NaH_2PO_4, pH 6.9 for 45 min. Cells were washed 2 x 5 min with PBS and extracted for 10 min with 0.5% Triton X-100 in PBS. 3) Cells were fixed in prechilled absolute methanol for 10 min at -20°C. Following all fixation protocols, cells were washed 3 x 5 min with PBS. Prior to staining, cells were washed 3 x 4 min with 2 mg/ml sodium borohydride in PBS to reduce free aldehyde groups and decrease background staining, followed by 3 x 4 min PBS rinses. All steps were done at room temperature (RT). Double immunofluoresence labelling was done sequentially at RT with all antibody incubations lasting 45 min. All antibody incubations were followed by 3 x 4 min rinses in PBS. Following the last set of PBS washes, cells were stained with 0.3 μg/ml Hoechst 33258 in PBS for 1 min to visualize DNA, washed 3 x 4 min with PBS, and mounted in 50% glycerol-PBS, pH 7.8 containing 0.1% (w/v) p-phenylene diamine. Samples were observed with a Zeiss Axiophot microscope with epifluorescence optics and photographed on Ilford XP1-400 ASA film.

The following primary antibodies were used: YOL 1/34, a rat monoclonal antibody which recognizes most forms of α-tubulin (Serotec, Mississauga, Ont.) (used at 1:10); HM2, a mouse monoclonal antibody which stains both high and low molecular weight isoforms of MAP2 (Sigma, St. Louis, MO) (diluted 1:400); 6-11B-1, a mouse monoclonal antibody against acetylated α-tubulin (Sigma) (used at 1:100); a rabbit polyclonal antibody against βIII tubulin (used at 1:100) was the kind gift of Dr. A.

Frankfurter; 9E10, a mouse monoclonal antibody that recognizes a 10 amino acid peptide from the human c-myc protein (Evan *et al.*, 1985) (used undiluted); a rabbit polyclonal antibody recognizing both HMW-MAP2 and MAP2c was a kind gift from Dr. Itzhak Fischer (diluted 1:1500); a rabbit polyclonal antibody against cow glial fibrillary acidic protein (GFAP) (used at 1:150) was obtained from Accurate Chemical and Scientific Corp. (Axell), Westbury, NY; and MF20, a mouse monoclonal antibody against chicken sarcomeric myosin (Developmental Studies Hybridoma Bank, University of Iowa) (used at 1:1000).

The following secondary antibodies were used: FITC-conjugated donkey anti-rat IgG (used at 1:100) (Jackson, BIO/CAN Scientific, Mississauga, Ont.), cross-adsorbed to mouse and rabbit; CY3-conjugated donkey anti-mouse IgG (used at 1:500) (Jackson), cross-adsorbed to rabbit and rat; FITC-conjugated goat anti-rabbit IgG (used at 1:100) (Sigma).

WESTERN BLOT ANALYSIS

Whole cell (WC) protein was extracted using the method of Drubin *et al.* (1985) from sets of neuronally induced untransfected and Tα1MAP2cmyc-transfected P19 cells grown in tissue culture, but a higher concentration of protease inhibitors was used in the lysis buffer (25 mM Na_2HPO_4, 0.4 M NaCl, 0.5% SDS, 40 μM benzamidine-HCl (Sigma), 4 mM p-aminoethylbenzene-sulfonyl fluoride (PEFA) (Centrichem Inc., Stamford CT), 2 mM 1,10-phenanthroline (Sigma), 40 μg/ml each of aprotinin, leupeptin, and pepstatin A (all from Sigma), pH 7.2) The cell suspension was heated in a boiling water bath for 10 min, centrifuged at 15,000 RPM for 10 min in a microspin 24 microfuge (DuPont, Mississauga, Ont.) and the supernatant collected. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein (30 μg) in sample buffer (Laemmli, 1970) were heated in a boiling water bath for 3 min and separated on 7.5% SDS polyacrylamide gels. The gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 10 min and transferred electrophoretically to nitrocellulose using a BIORAD transblot system. The blots were blocked for 1 hour with 5% skim milk powder in PBS prior to a 1 hour incubation in primary antibody. Primary antibodies used were: HM2 (described above), diluted 1:2000; 9E10 (described above), diluted 1:2. The blots were washed 3 x 5 min with 5% skim milk in PBS and incubated for 45 min in biotinylated-horse anti-mouse IgG (Dimension Labs Inc., Mississauga, Ont.) diluted 1:1000. Following 3 x 5 min PBS washes, the filters were incubated for 30 min with streptavidin horseradish peroxidase (S-HRP) (Amersham, Oakville, Ont.) diluted 1:5000 in PBS. After 3 x 5 min PBS washes, the ECL system was used to visualize the reactive proteins (Amersham). All antibodies were diluted with 2% skim milk in PBS. All washes and incubations were performed at RT.

ENZYME-LINKED IMMUNOSORBENT ASSAY

WC protein was harvested as described above from sets of untransfected and Tα1MAP2cmyc-transfected P19 neuronal differentiation timecourses and assayed using the modified method of Voller *et al.* (1979). Equal amounts of protein (15 μg) for each day of differentiation were loaded in triplicate onto 96-well Xenobind covalent binding microwell plates (Xenopore Corp., Hawthorne NJ). Prior to loading, protein samples were diluted to a final volume of 50 μl with PBS. A serial dilution of crude bovine brain protein extract, made in PBS, was used as a MAP2 standard for relative MAP2 protein level determinations. Protein samples were placed in a dry, 37°C incubator overnight. Samples were blocked at RT for 30 min with 5% skim milk, 0.05% Tween 20 in PBS. The blocking buffer was replaced with 50 μl of the primary antibody HM2, diluted 1:1000 in antibody buffer (2.5% skim milk, 0.05% Tween 20 in PBS) and samples were incubated covered at 37°C for 1 hour, followed by 3 x 3 min washes with antibody buffer. The samples were incubated covered at 37°C for 1 hour with 50 μl of biotinylated-goat anti-mouse IgG (Amersham), diluted 1:1000 in antibody buffer and followed by 3 x 3 min washes with 0.2% Tween 20 in PBS. 50 μl of S-HRP, diluted 1:750 in PBS, was added and samples were sealed and incubated at 37°C for 45 min. Samples were washed 3 x 3 min with 0.2% Tween 20 in PBS and 100 μl of o-phenylenediamine (Sigma) substrate was added and incubated in darkness for 10 min. An equal volume of 1 N H_2SO_4 was added to each well to stop the reaction. Absorbance was read at 490 nm using a Ceres UV900HDi scanning autoreader (Bio-Tek Instruments Inc., Winooski VT).

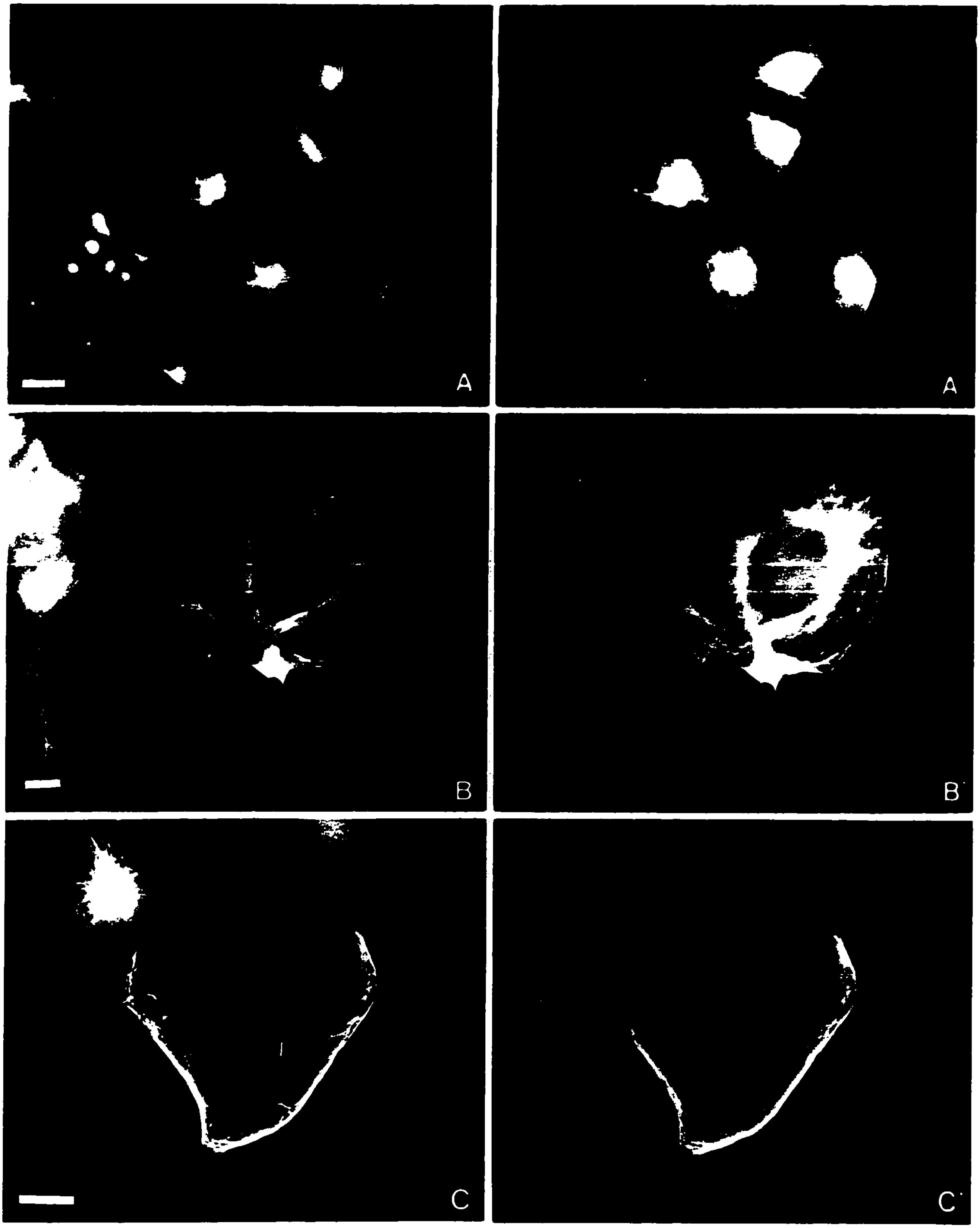
RESULTS

MICROTUBULE REARRANGEMENT IN P19 CELLS TRANSIENTLY TRANSFECTED WITH MAP2C AND HMW-MAP2

The effect of inappropriate MAP2c and HMW-MAP2 expression was investigated by transiently transfecting undifferentiated P19 cells with plasmids containing the coding sequences for these two proteins. Figures 5 and 6 show several fields of P19 cells transiently transfected with MAP2c and HMW-MAP2, respectively. Transfected cells are identified throughout by their positive staining with an antibody against MAP2 (Fig. 5 A′-E′ and Fig. 6 A′-C′). An anti-α-tubulin antibody reveals the MT organization of both transfected and untransfected cells in these same fields (Fig. 5 A-E and Fig. 6 A-C).

Several types of MT arrangements were observed among the population of cells inappropriately expressing either MAP2 protein. Many transfected cells had unaltered, centrosome-based, radial MT arrays composed of MAP2c-decorated MTs (Figs. 5 A,A′ and 6 A,A′). The MT organization of these cells and untransfected cells appeared indistinguishable. However, MT bundles were also observed in many transfected P19 cells (Figs. 5 B,C and 6 B,C). Like the unaltered MT arrays, MT bundles showed positive MAP2 staining while the background, untransfected cells were not stained with the anti-MAP2 antibody (Figs. 5 B′,C′ and 6 B′,C′). Although MT bundles were formed, centrosome-based MTs persist in many of these cells (Fig. 5 C). In some bundle-

Figure 5. Double immunofluorescence staining of P19 cells transiently-transfected with pPGK2c and prepared by the simultaneuos fixation/extraction method. A-E stained for α-tubulin using YOL1/34 and A′-E′ for MAP2 using HM-2. F,F′ secondary antibody controls. Bars in A,F (valid for all except B-C′)=20μm Bars in B,C (valid for B-C′)=10μm


A
A
B
B'
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C'

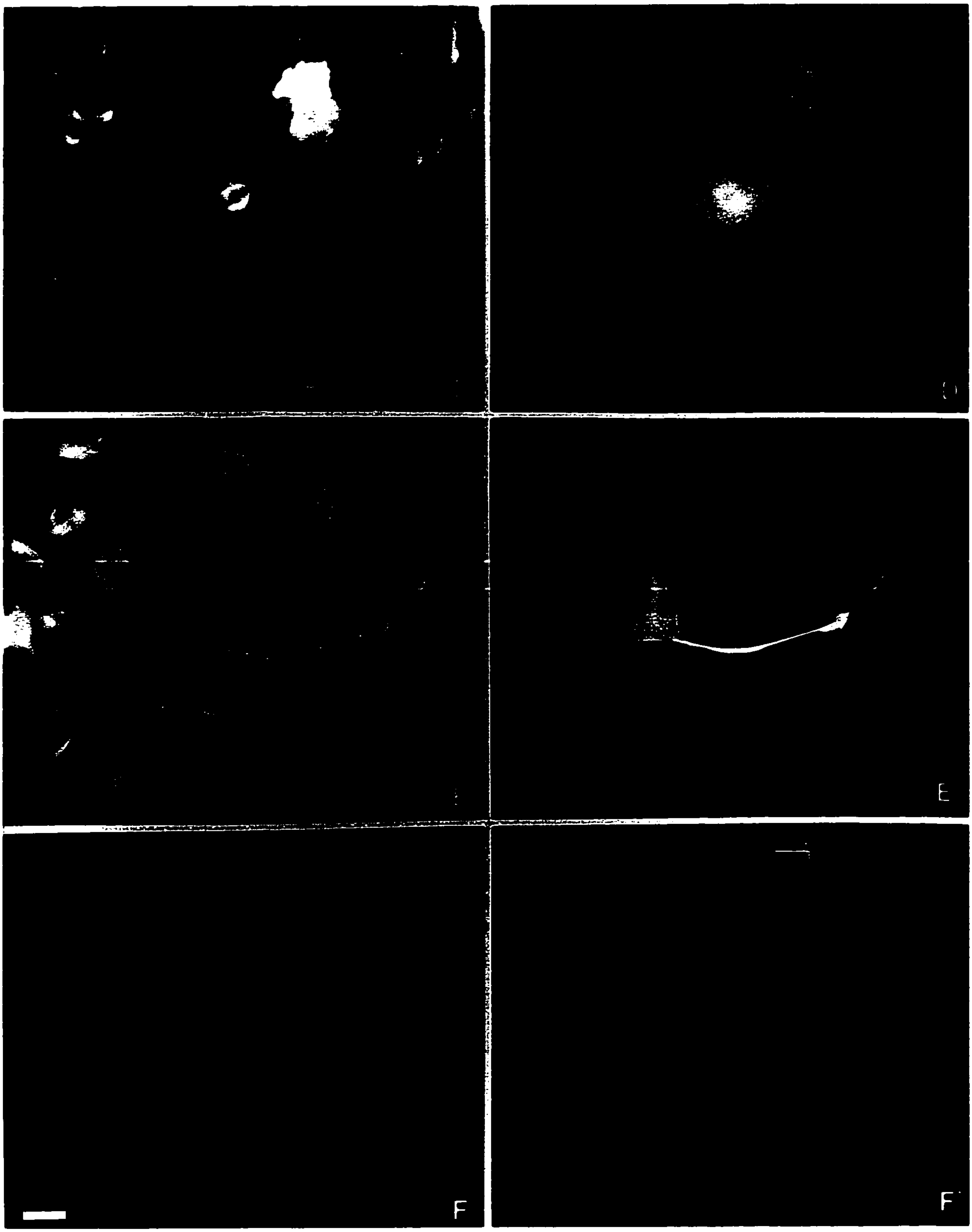
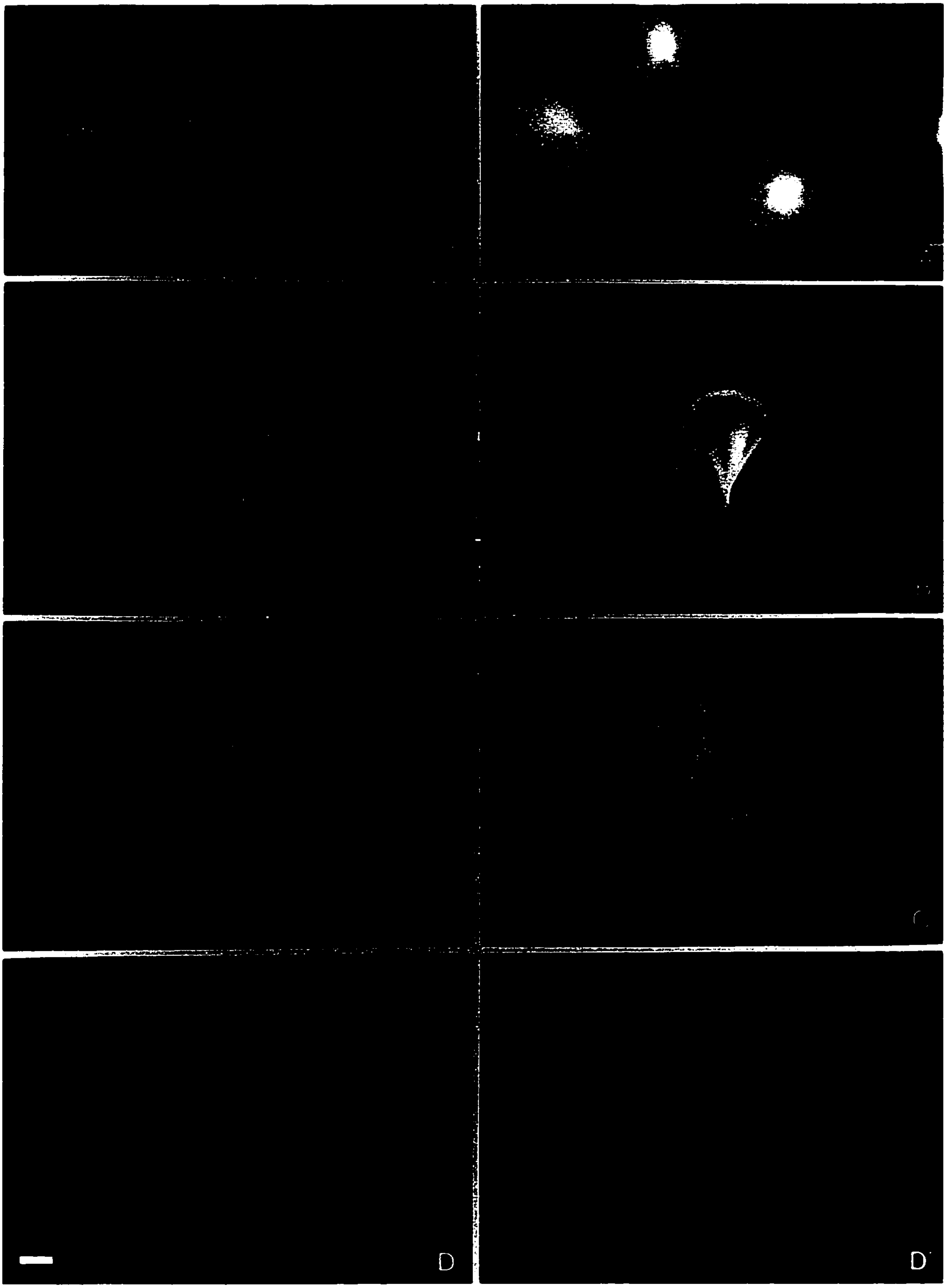

D
E
F
F'

Figure 6. Double immunofluorescence staining of P19 cells transiently-transfected with pPGK2b and prepared by the simultaneous fixation/extraction method. A-C stained for α-tubulin using YOL1/34 and A′-C′ for MAP2 using HM-2. D,D′ secondary antibody control. Bar=10μm


B'
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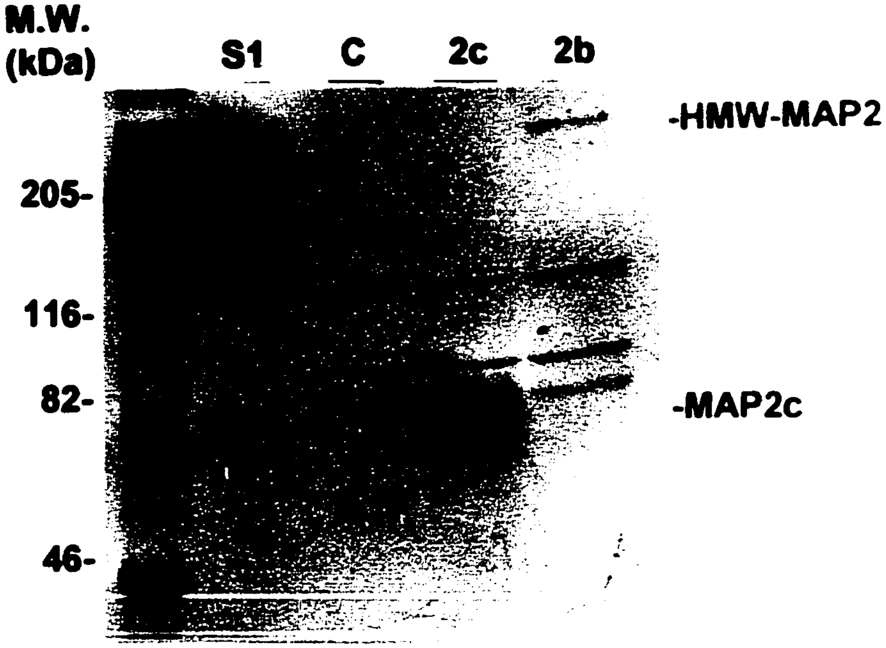
D'

containing cells, short cytoplasmic extensions were observed (Figs. 5 B and 6 C), while MT rings were predominant in others (Figs. 5 C and 6 B). Occasionally, mitotic, MAP2c- and HMW-MAP2-expressing cells were seen but their occurrence in a population of transfected cells was rare (Fig. 5 D,D′). The spindle apparatus of these cells appeared normal (Fig. 5 D) but it is unclear, due to the high level of cytoplasmic MAP2c staining, if the MTs were decorated with MAP2c (Fig. 5 D′). In a small proportion of transfected cells, long cytoplasmic processes, reminiscent of developing neurites and containing densely packed MTs, were observed (Fig. 5 E,E′). Figure 5 (F,F′) and Figure 6 (D,D′) demonstrate the specificity of the secondary antibodies by showing negligible background staining when primary antibody incubations were replaced with PBS incubations.

Another observation common to both MAP2c and HMW-MAP2 transfections was the infrequent occurrence of transfected cell clusters. The majority of transfected cells were found isolated from one another, with the exception of small groups of cells whose MT organization appeared unaltered and similar to untransfected cells. The MAP2 staining intensity of these cells was lower than in transfected cells possessing bundled MTs. The degree of MT bundling in transfected cells appeared to increase as MAP2 staining intensity increased. The proportion of transfected cells possessing MT bundles was slightly higher for MAP2c (47%) than HMW-MAP2 (38.7%).

By immunofluorescence microscopy, MAP2c was detected in a greater percentage of cells (10-15%) compared to HMW-MAP2 transfected cells (less than 1%). Western blotting of whole cell (WC) protein extracts from populations of transfected cells confirmed this observation (Fig. 7). At least two bands were detected in protein extracts from MAP2c-transfected cells (lane 2c) with the upper band co-migrating with MAP2c from bovine brain (lane S1). The other MAP2c band showed greater electrophoretic mobility. In some preparations of untransfected, control P19 extracts (lane C), small amounts of MAP2c were detected. Protein extracts from HMW-MAP2 transfected cells (lane 2b) contained small amounts of HMW-MAP2 which appeared as a faint band co-migrating with HMW-MAP2 from crude brain extract (lane S1). The extra bands that appear in lanes C, 2c, and 2b appear to be due to non-specific binding of the anti-MAP2 antibody to protein in P19 extracts. These extra bands were detected at equal intensity in all P19 protein extracts, including extracts from untransfected cells, but did not interfere with the immunofluorescence staining observations (refer to Figs. 5 and 6). The HM-2 anti-MAP2 antibody only labelled MTs in transfected cells since neighbouring, untransfected were not stained by this antibody.

Figure 7. Western blot of MAP2 expression in transiently-transfected P19 cells. Whole cell extracts from untransfected (C), MAP2c (2c) and HMW-MAP2 (2b) transfected P19 cells were immunoblotted with HM2. Crude brain extract (S1) was used as a MAP2 standard. Molecular weight standards are indicated to the left of the blot.


M.W.
(kDa)
S1
C
2c
2b
-HMW-MAP2
205-
116-
82-
-MAP2c
46-

MICROTUBULE STABILITY IN P19 CELLS TRANSIENTLY TRANSFECTED WITH MAP2C AND HMW-MAP2

Changes in the MT stability of MAP2c and HMW-MAP2 transiently transfected P19 cells were assayed in two ways. Acetylation, one of the posttranslational modifications that occurs on α-tubulin, is generally thought to be a marker for increased MT stability (Piperno *et al.*, 1987). As a first method in assaying MT stability, double-labelling with anti-acetylated tubulin and anti-MAP2 antibodies was performed for both untransfected and transfected cells (Figs. 8 and 9). P19 cells transfected with MAP2c (Fig. 8 A′) or HMW-MAP2 (Fig. 9 A′) typically show MT bundles intensely labelled for acetylated α-tubulin (Fig. 8 A, arrow and Fig. 9 A, arrowhead). On the other hand, untransfected cells, revealed by DNA staining (Fig. 8 A′′ and Fig. 9 A′′), exhibit, at best, only faint acetylated tubulin labelling (Fig. 8 A and Fig. 9 A). When observed, acetylated α-tubulin staining in untransfected cells was restricted to midbodies. Some transfected cells (Fig. 9 A, A′, arrow) did not possess acetylated MT bundles but, instead, resembled untransfected cells with anti-acetylated α-tubulin staining restricted to midbodies. The MAP2 staining in these cells was generally less intense than in transfected cells containing MT bundles (Fig. 9 A′).

As a second method to assess changes in MT stability, transfected cells were treated with the MT depolymerizing drug colchicine and later stained for immunofluorescence microscopy.

Figure 8. Double immunofluorescence staining of acetylated, stable MT bundles in P19 cells transiently-transfected with pPGK2c and prepared by the precipitative method. A-B stained for acetylated α-tubulin using 6-11B-1, A′-B′ for MAP2 using HM-2, and A′′-B′′ for DNA. A-A′′ are untreated, MAP2c-transfected cells possessing acetylated MT bundles (arrow). B-B′ is a transfected cell, treated with colchicine for 2 hours, possessing acetylated MT bundles (arrow). Bar=20μm

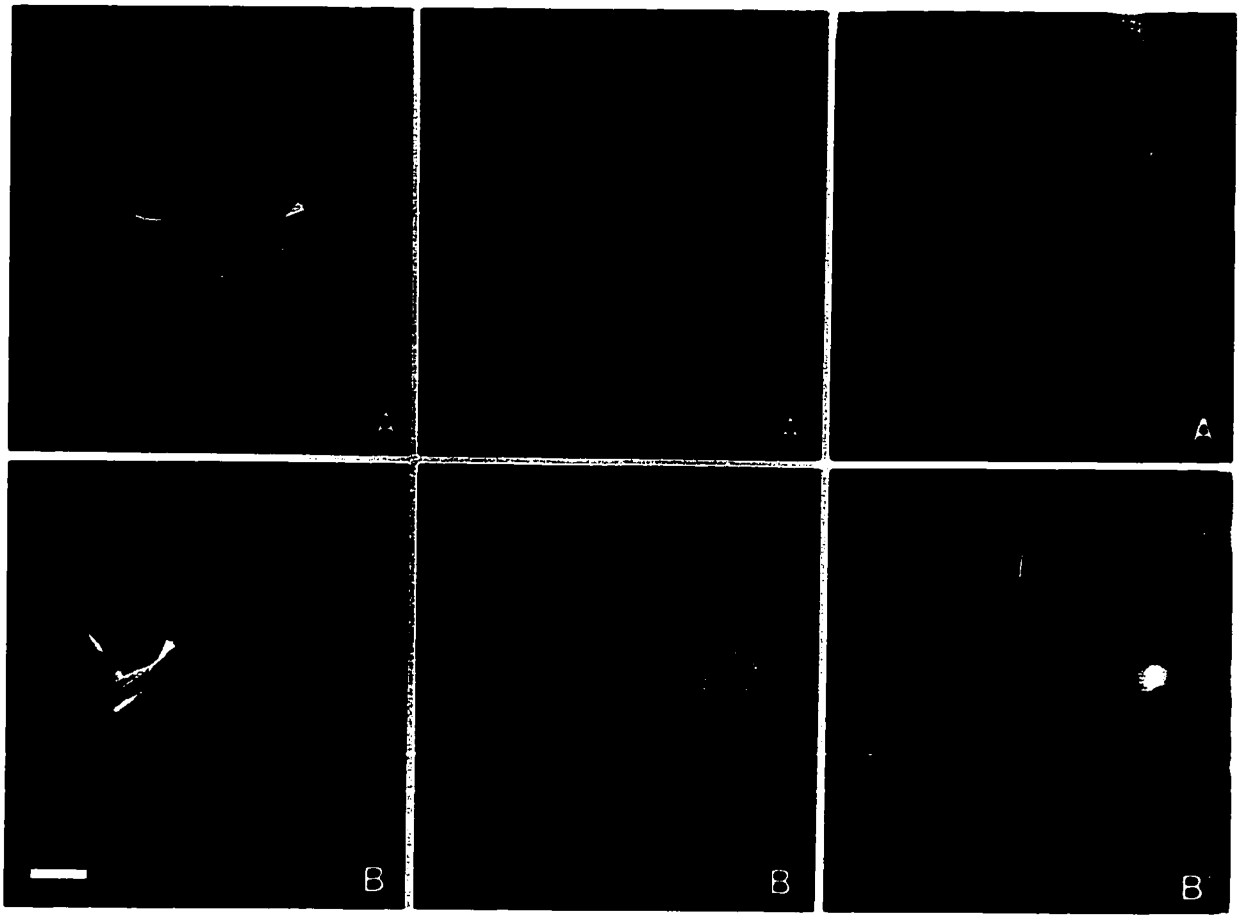
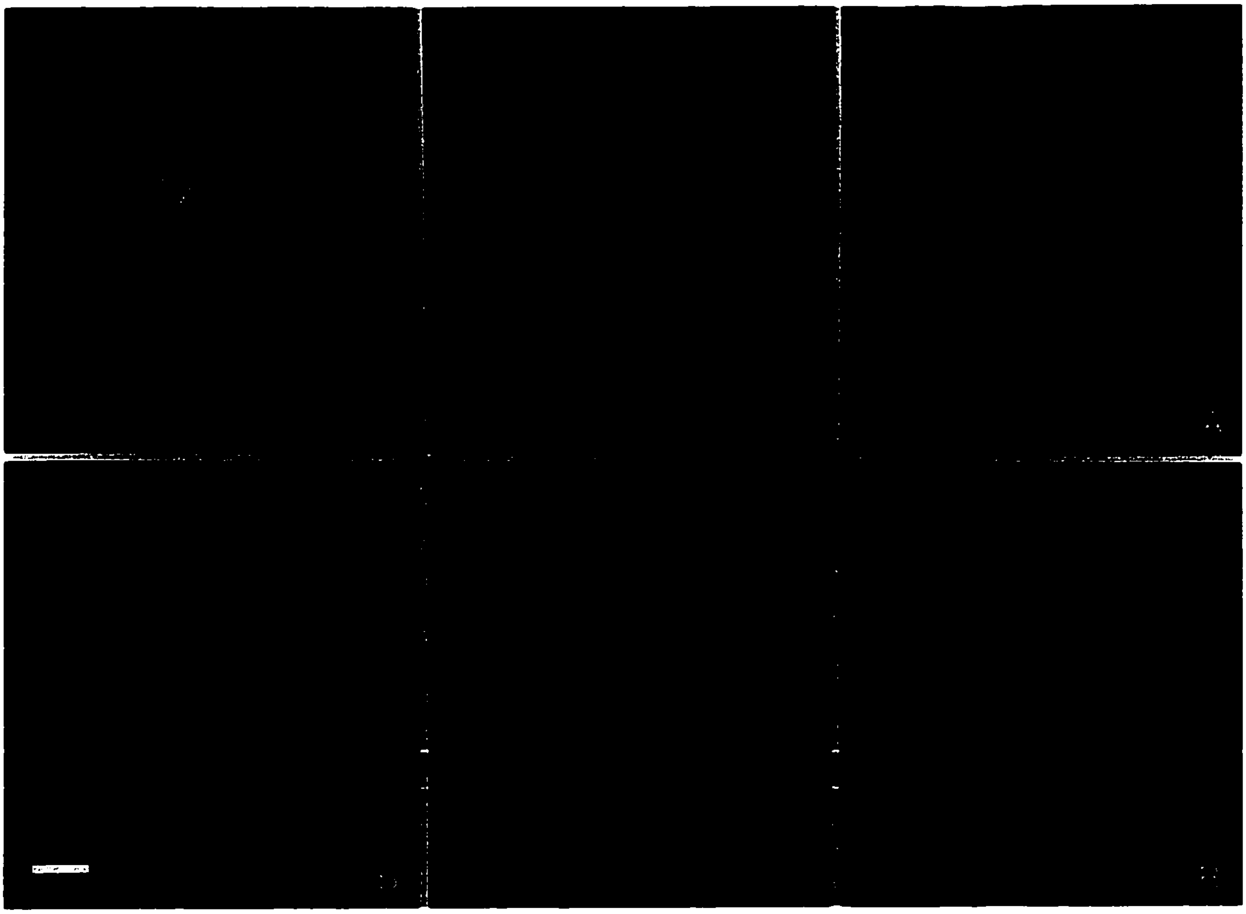

A
A
A
B
B
B

Figure 9. Double immunofluorescence staining of acetylated, stable MT bundles in P19 cells transiently-transfected with pPGK2b and prepared by the precipitative method. A-B stained for acetylated α-tubulin using 6-11B-1, A′-B′ for MAP2 using HM-2, and A′′-B′′ for DNA. A-A′′ are untreated, HMW-MAP2-transfected cells possessing acetylated MT bundles (arrowhead) and more dimly MAP2 stained cells lacking acetylated MT bundles (arrow). B-B′′ is a transfected cell, treated with colchicine for 2 hours, possessing acetylated MT bundles. Bar=20μm



Anti-acetylated α-tubulin staining was observed only on the colchicine-resistant MT bundles of MAP2c- (Fig. 8 B, arrow) and HMW-MAP2- (Fig. 9 B) transfected cells. The surrounding, untransfected cells, seen in Figures 8 B'' and 9 B'', did not stain with the anti-acetylated α-tubulin antibody (Figs. 8 B and 9 B). Even the midbodies, frequently seen in untreated cells, were absent in cells exposed to colchicine.

Transfected cells, treated with colchicine for various lengths of time, were also double-labelled with an anti-MAP2 and the anti-tubulin antibody YOL1/34. P19 cells transfected with MAP2c (Fig. 10 A') or HMW-MAP2 (Fig. 11 A') showed a variety of MT arrangements (Figs. 10 A and 11 A), as previously described. After colchicine treatment, only transfected cells possessed stable MTs, which were usually bundled (Figs. 10 B and 11 B, arrows). The number and size of stable MT bundles, in cells transfected with either MAP2 construct, decreased as the duration of colchicine exposure increased. However, for both sets of transfections, stable MT bundles were seen in some transfected cells after all colchicine exposure times assayed.

To compare the MT stabilizing abilities of MAP2c and HMW-MAP2, the percentage of transfected cells containing at least one stable MT bundle was determined for each time point assayed. For each protein, values from three experiments, performed on different days, were plotted and the slope shown is the regression line for the three experiments (Fig.12). For both sets of transfection experiments, the percentage of transfected

Figure 10. Double immunofluorescence staining of P19 cells transiently-transfected with pPGK2c and prepared by the precipitative fixation method. A-B stained for α-tubulin using YOL1/34, A′-B′ for MAP2 using HM-2, and A′′-B′′ for DNA. A-A′′ are untreated, MAP2c-transfected cells. B-B′′ are transfected cells treated with colchicine for 2 hours, possessing stable MT bundles (arrow). Bar=20μm

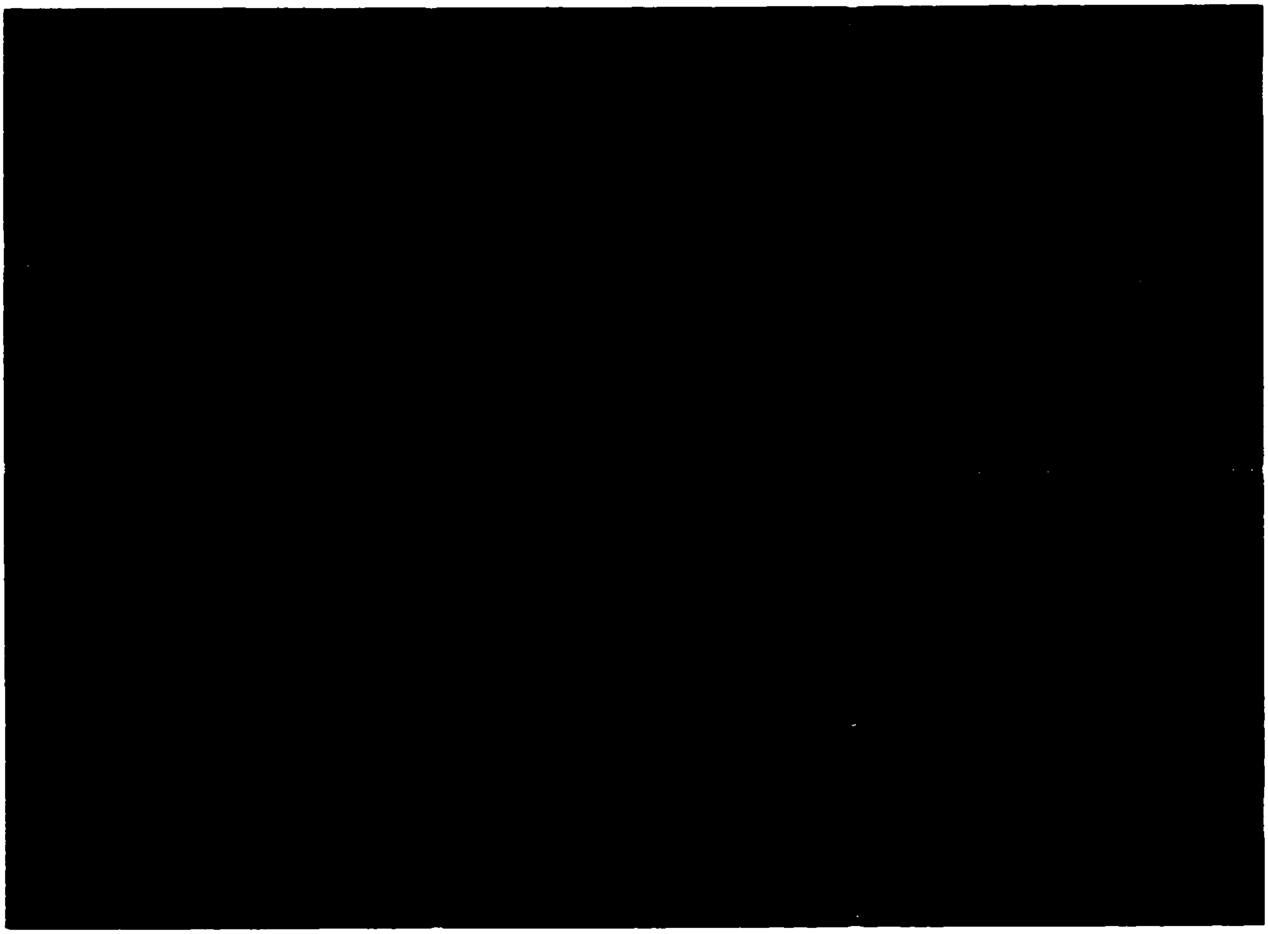


Figure 11. Double immunofluorescence staining of P19 cells transiently-transfected with pPGK2b and prepared by the precipitative fixation method. A-B stained for α-tubulin using YOL1/34, A′-B′ for MAP2 using HM-2, and A′′-B′′ for DNA. A-A′′ are untreated, HMW-MAP2-transfected cells. B-B′′ is a transfected cell treated with colchicine for 4 hours, possessing stable MT bundles (arrow). Bar=20μm

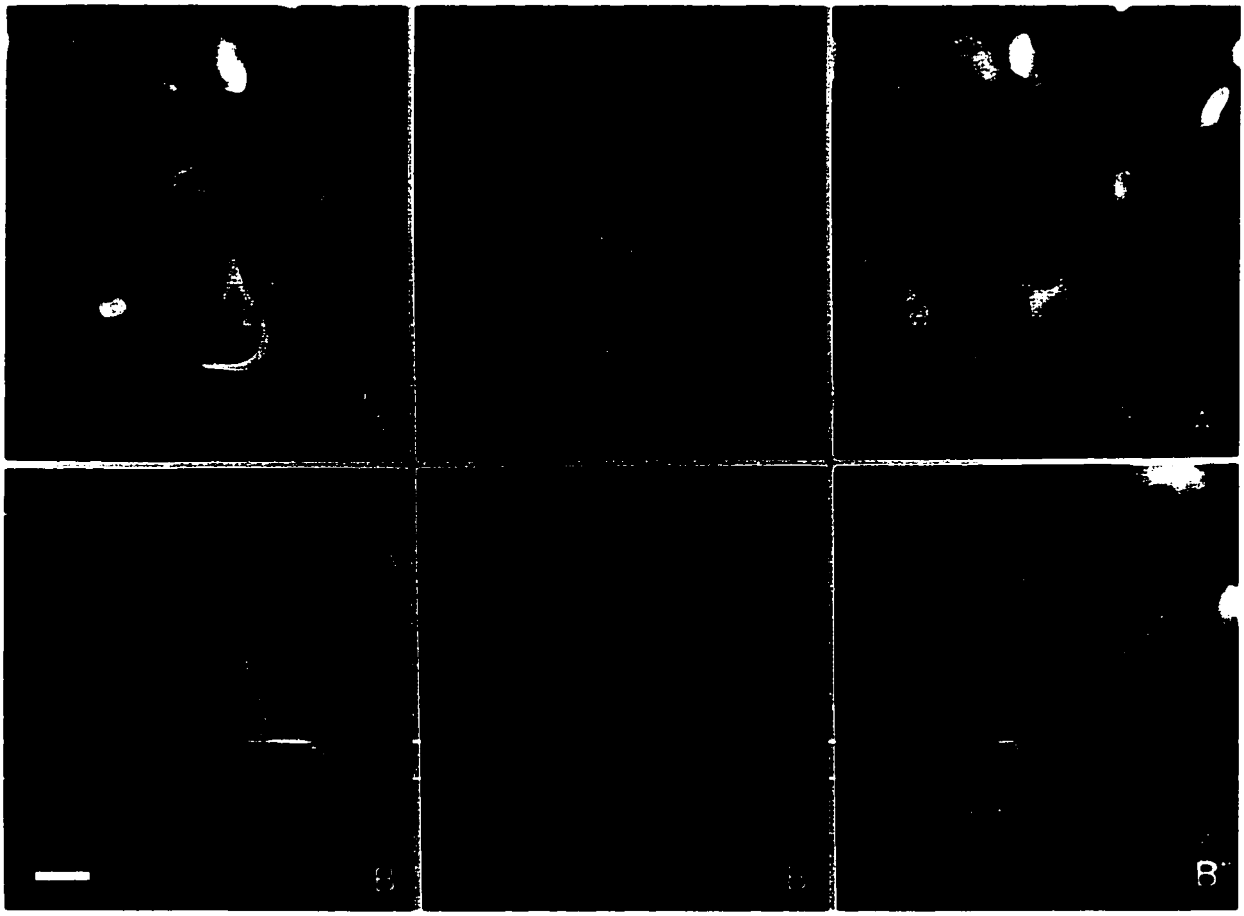
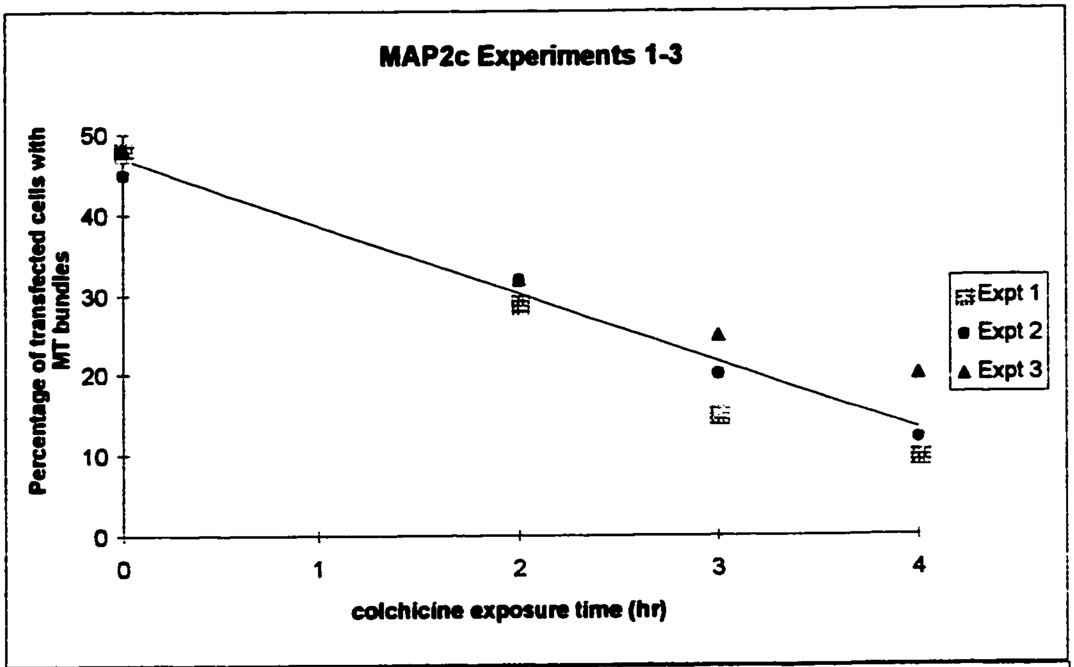
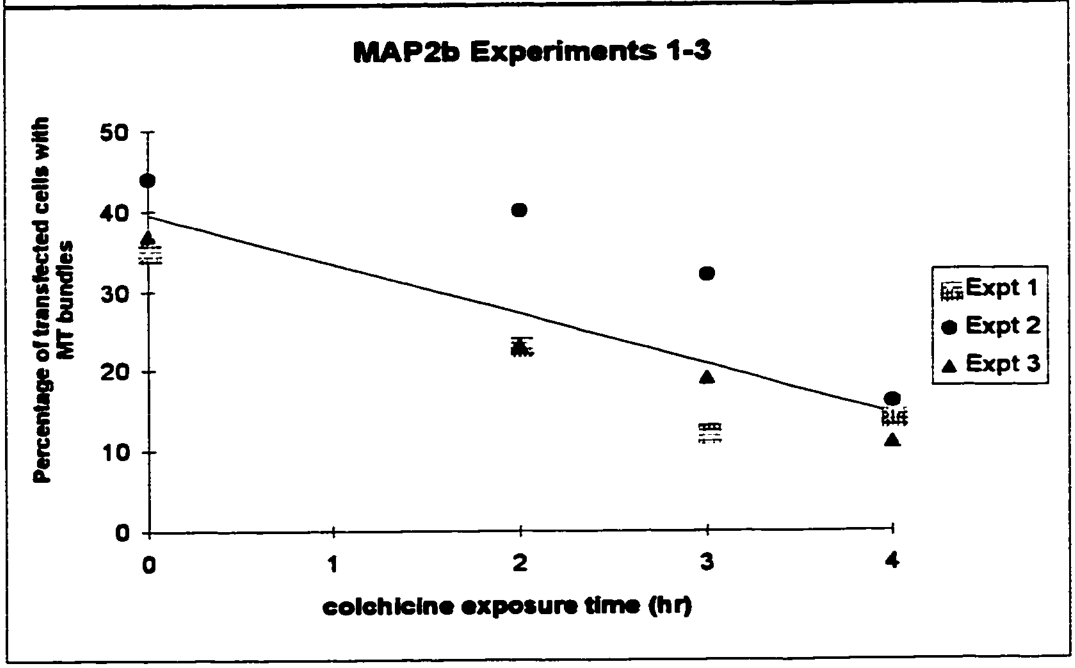

A
B
B''

Figure 12. MT stability comparison in P19 cells transiently-transfected with MAP2c and HMW-MAP2. Cells expressing MAP2c and HMW-MAP2 were exposed to colchicine for various lengths of time, fixed, stained for α-tubulin using YOL1/34 and MAP2 using HM-2, and observed by immunofluorescence microscopy. At each time point, the percentage of transfected cells containing at least one stable MT bundle was determined. Individual values from three experiments were plotted for each protein.



MAP2c Experiments 1-3



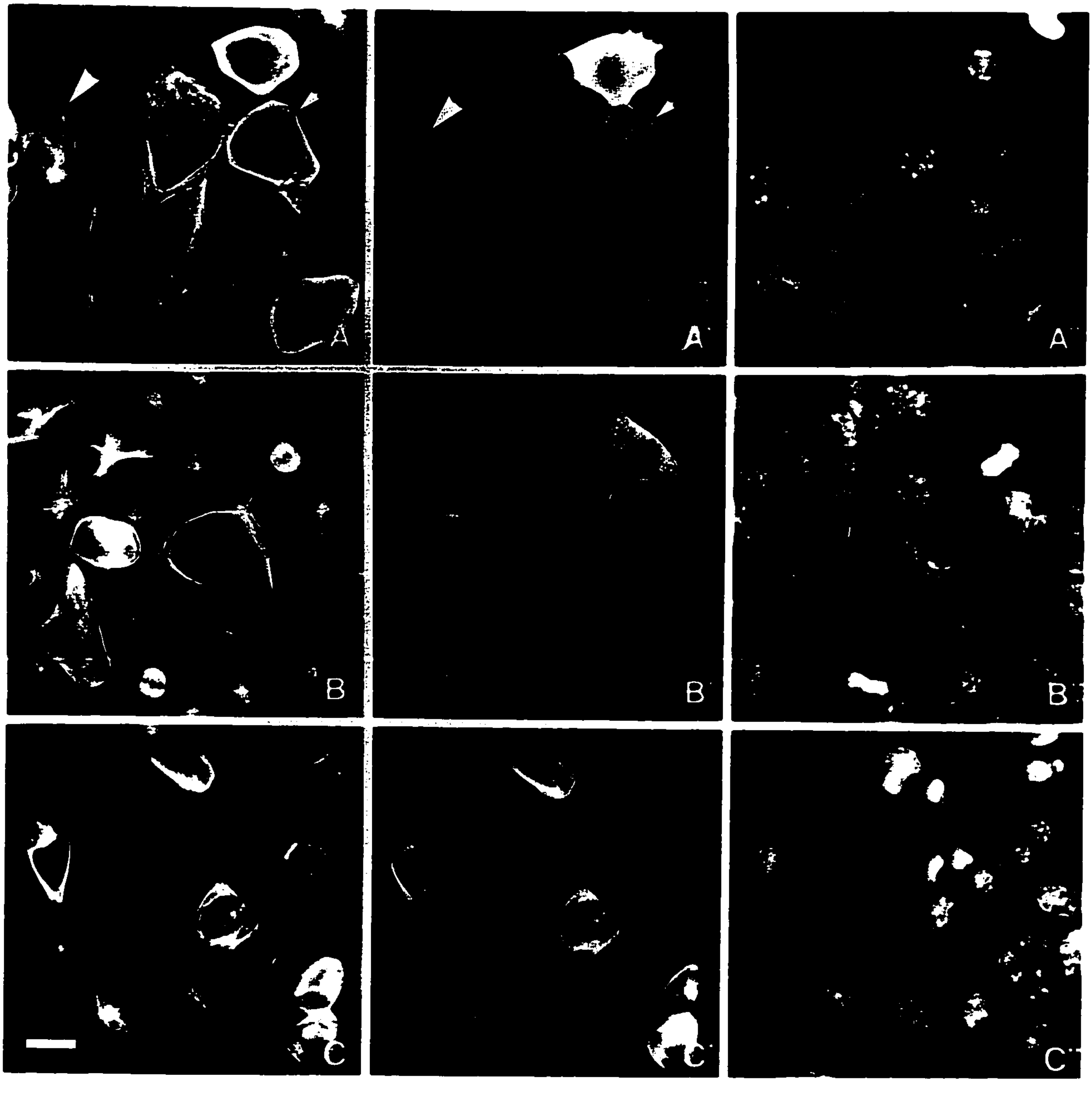
MAP2b Experiments 1-3

cells with stable MT bundles decreased linearly with the duration of colchicine treatment. However, experiment #2 performed with MAP2b had outlying values at 2 and 3 hours of colchicine exposure which resulted in greater sample variance within the set of MAP2b transfection experiments. The slopes of the curves for MAP2c and MAP2b were not significantly different as determined by the analysis of covariance (ANCOVA) test (F=2.2, residuals mean-square=30, p=0.16, n=24), suggesting that these two proteins stabilize MTs to similar extents.

COMPARISON OF MAP2C AND MYC-TAGGED MAP2C EXPRESSION IN TRANSIENTLY TRANSFECTED P19 CELLS

To test whether a myc-epitope tag on the carboxy-terminus of MAP2c affects its ability to reorganize MTs, MAP2c and MAP2cmyc were transiently transfected into undifferentiated P19 cells and their effects on MT organization were compared. Figure 13 (A, A′, A′′) shows a typical field of P19 cells transiently transfected with MAP2c. As previously described, normal MT organization (arrowhead) and MT bundling (arrow) were observed. P19 cells, transfected with myc-tagged MAP2c, showed similar MT arrangements (Fig. 13 B, B′, B′′), demonstrating that a myc tag at the carboxyl-terminus of MAP2c does not affect its binding to or bundling of microtubules. This result indicated that MAP2cmyc could be used in stable transfection studies where its expression, in differentiating neurons, could be distinguished from endogenous MAP2c expression.

Figure 13. Double immunofluorescence staining of P19 cells transiently-transfected with pPGK2c (A′-A′′ and C-C′′) and pPGK2cmyc (B-B′′) and prepared by the precipitative fixation method (A-A′′ and B-B′′) and the simultaneous fixation/extraction method (C-C′′). A-C stained for α-tubulin, A′,C′ for MAP2, B′ for the myc-epitope tag, and A′′-C′′ for DNA. Bar=20μm

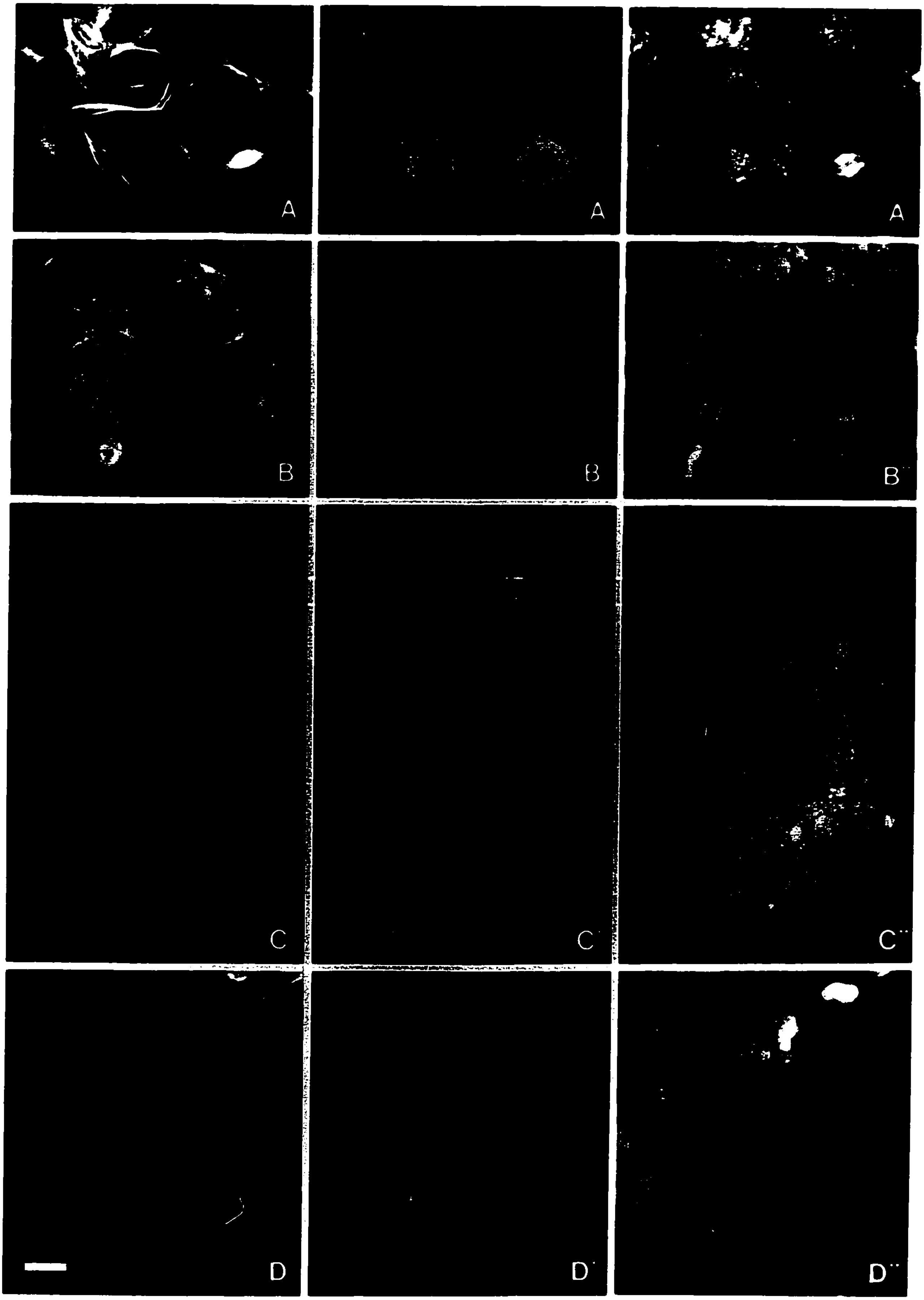

A
A
A
B
B
B
C
C
C

To determine if all MAP2 protein was associated with the MTs of transfected cells, two fixation methods were used to prepare cells before staining. The precipitative method, which preserves the soluble, cytoplasmic protein component, revealed MAP2c and MAP2cmyc staining in the cytoplasm of transfected cells (Fig. 13 A′ and B′). The simultaneous fixation/extraction method, which removes soluble proteins from the cytoplasm, showed MAP2c (Fig. 13 C, C′) and MAP2cmyc (results not shown) associated with MTs. These results demonstrate that not all transfected MAP2 protein is bound to MTs but is present as soluble protein in the cytoplasm.

EXPRESSION OF Tα1-MAP2CMYC IN STABLY TRANSFECTED P19 CELLS

To examine the effects of inappropriate MAP2c expression on neuronal differentiation, P19 cells were first transiently transfected with myc-tagged MAP2c and then induced to differentiate along the neuronal pathway. Before neuronal induction, typical MT rearrangements, previously observed with MAP2c, were seen in cells expressing myc-tagged MAP2c (Fig. 14 A, A′). One day following RA-induction, transfected cells were still observed and resembled transfected cells in uninduced cultures (Fig. 14 B, B′). Untransfected and transfected mitotic cells were also seen in these cultures (Fig. 14 B, B′, B′′). BIII-tubulin staining, used to detect neurons in these cultures, was first observed two days after neuronal induction but was absent or only weakly detected (Fig. 14 C) in cells expressing

Figure 14. Double immunofluorescence staining of neuronally-induced P19 cells, transiently-transfected with MAP2cmyc and prepared using the simultaneous fixation/extraction method. A-B stained for α-tubulin using YOL1/34, C-D for class III ß-tubulin, A′-D′ for the myc-epitope tag using 9E10, and A′′-D′′ for DNA. A-A′′ are undifferentiated cells (Day 0), B-B′′are cells one day following RA-induction (Day 1), C-C′′are Day 2 cells, and D-D′′ are Day 3 cells. Bar=20μm

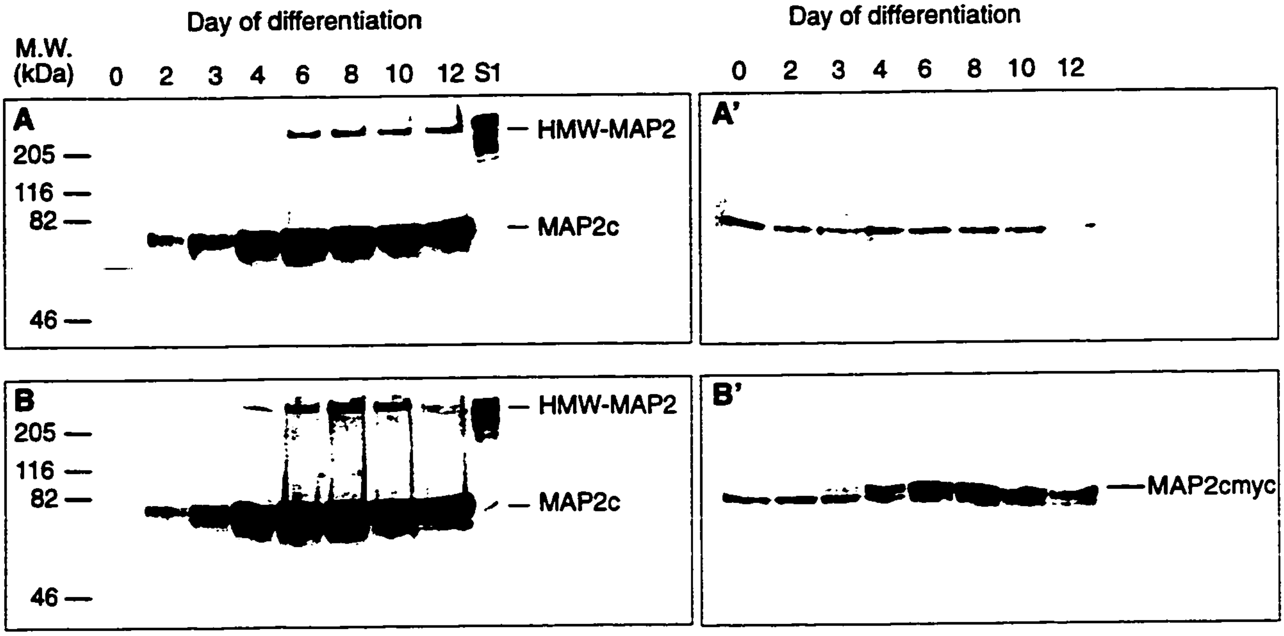

A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''

myc-tagged MAP2c (Fig. 14 C′). In addition, the number of cells expressing the transgene was reduced in these cultures, when compared with uninduced cultures. With rare exceptions, transgene expression was restricted to cells in aggregates. Three days after RA-induction, the occasional transfected cell was observed among developing neurons (Fig. 14 D, D′). In general, these cells lacked neuritic processes and were, at best, poorly labelled for βIII-tubulin.

To investigate the effects of MAP2c overexpression during neuronal differentiation, the developmentally regulated Tα1 α-tubulin promoter was used to drive neuronal-specific expression of MAP2cmyc in differentiating P19 cells. Figure 15 shows Western blots in which equal amounts of protein, from one set of control P19 and stably-transfected MAP2cmyc cultures, were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with antibodies to MAP2 and human c-myc epitope. MAP2 protein accumulation patterns are similar for both P19 and Tα1-MAP2cmyc cell lines induced to differentiate along the neuronal pathway (Fig. 15 A and B). MAP2c was first detected 2 days after RA induction. The level of MAP2c protein increased as the neuronal cultures matured, peaked at approximately day 8, and then decreased slightly in the day 10 and 12 samples. High molecular weight MAP2 (HMW-MAP2) first appeared four to six days after the addition of RA and also appeared to peak around day 8.

MAP2cmyc expression mimicks that of endogenous MAP2c (compare Fig. 15 B and B′) and no myc-tagged MAP2c was detected

Figure 15. Western blot of MAP2 and MAP2cmyc expression in neuronally-induced, untransfected and MAP2cmyc-stably-transfected P19 cells. Whole cell protein extracts from untransfected (A,A′) and Tα12cmyc-transfected (B,B′) P19 neuronal cultures were blotted for MAP2 using HM-2 (A,B) and myc using 9E10 (A′,B′). A crude brain extract (S1) was used as a MAP2 standard.

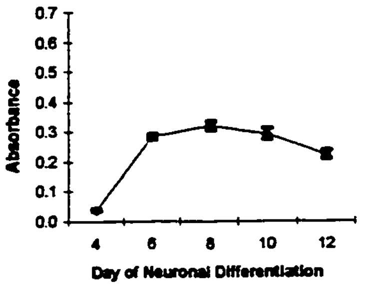

Day of differentiation
M.W. (kDa)
0
2
3
4
6
8
10
12
S1
A
205
116
82
46
HMW-MAP2
MAP2c
B
205
116
82
46
HMW-MAP2
MAP2c
Day of differentiation
0
2
3
4
6
8
10
12
A'
B'
MAP2cmyc

in control P19 cultures (Fig. 15 A′). The band, which migrated just below MAP2cmyc (Fig. 15 B′), also appeared in the myc blots for control P19 cultures (Fig. 15 A′) and was due to the nonspecific binding of the anti-myc antibody to a component in P19 WC protein extracts. By Western blotting, myc-tagged MAP2c was first detected as a faint band three days following RA addition (Fig. 15 B′) which is one day later than the appearance of endogenous MAP2c (Fig. 15 B). The amount of MAP2cmyc increased steadily as neuronal differentiation proceeded, peaked at approximately day 8, and decreased slightly in the day 10 and 12 samples.

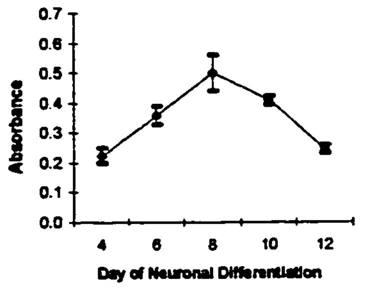
Although the Western blots showed that MAP2 temporal expression patterns were similar for transfected and control P19 cultures, the amount of MAP2c protein at each day of differentiation appeared to be greater for the Tα1-MAP2cmyc cell line (Fig. 15 A and B). To examine this further, ELISA was used for a relative quantification of total MAP2 protein from these same whole cell extracts (Figs. 16 and 17). In agreement with the Western blotting results, the pattern of MAP2 expression was similar for RA-induced cultures of control P19 and Tα1-MAP2cmyc cell lines. However, the Tα1-MAP2cmyc cultures accumulated more total MAP2 protein than untransfected P19 cultures for all test days. This difference was significant at Day 6, 8, and 10 as indicated by the student's t-test when $p<0.05$ (Day 4, $p=0.0535$, Day 6, $p=0.0008$, Day 8, $p=0.003$, Day 10, $p=0.00003$, and Day 12, $p=0.5371$). Figure 16 demonstrates the variability encountered

Figure 16. Relative quantification of total MAP2 protein in neuronally-induced untransfected (P19) and MAP2cmyc-stably-transfected (P19 2cmyc) P19 cells. Equal amounts of whole cell protein from each sample were analyzed by ELISA, using the anti-MAP2 antibody (HM-2). Each graph represents a separate experiment and each point is the mean absorbance for three replicates of each sample. Standard deviation is shown.

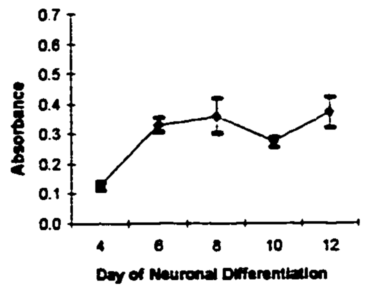
P19 Experiment 1



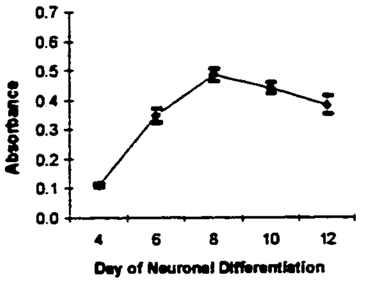
P19 Experiment 2



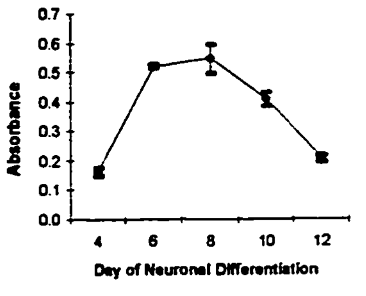
P19 Experiment 3



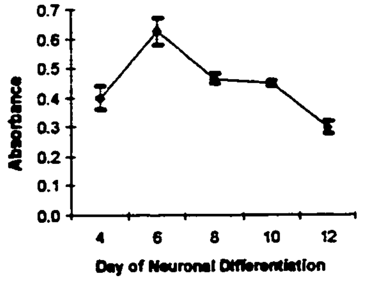
P19 2cmyc Experiment 1



P19 2cmyc Experiment 2



P19 2cmyc Experiment 3



P19 2cmyc Experiment 4

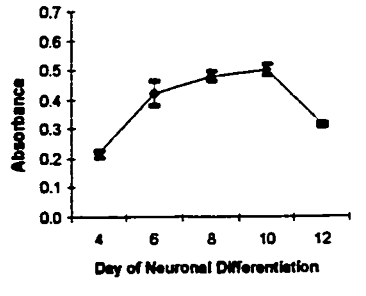
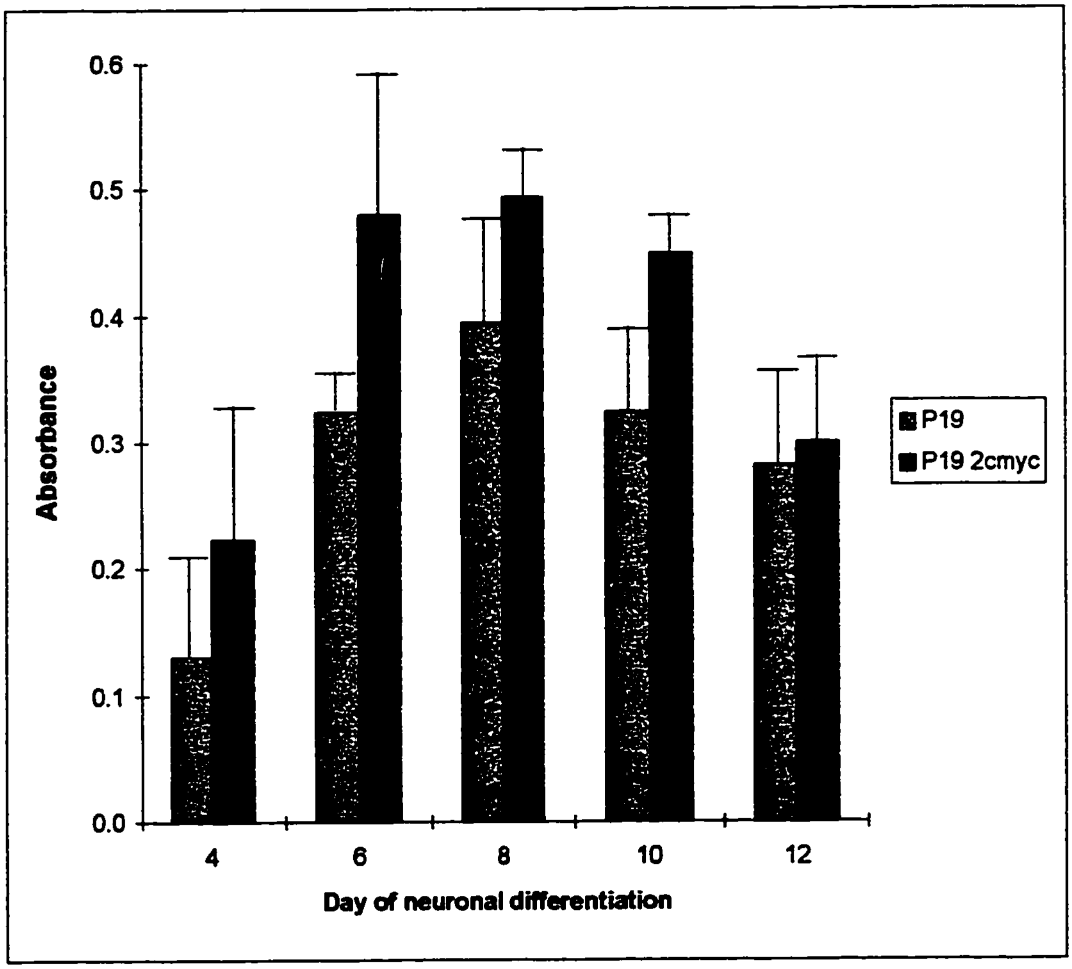


Figure 17. Relative quantification of total MAP2 protein in neuronally-induced untransfected (P19) and MAP2cmyc-stably-transfected (P19 2cmyc) P19 cells. Equal amounts of whole cell protein from each sample were analyzed by ELISA, using the anti-MAP2 antibody (HM-2). Each bar represents the mean absorbance value of three experiments with P19 cultures and four experiments with P19 2cmyc cultures. Standard deviation is shown.

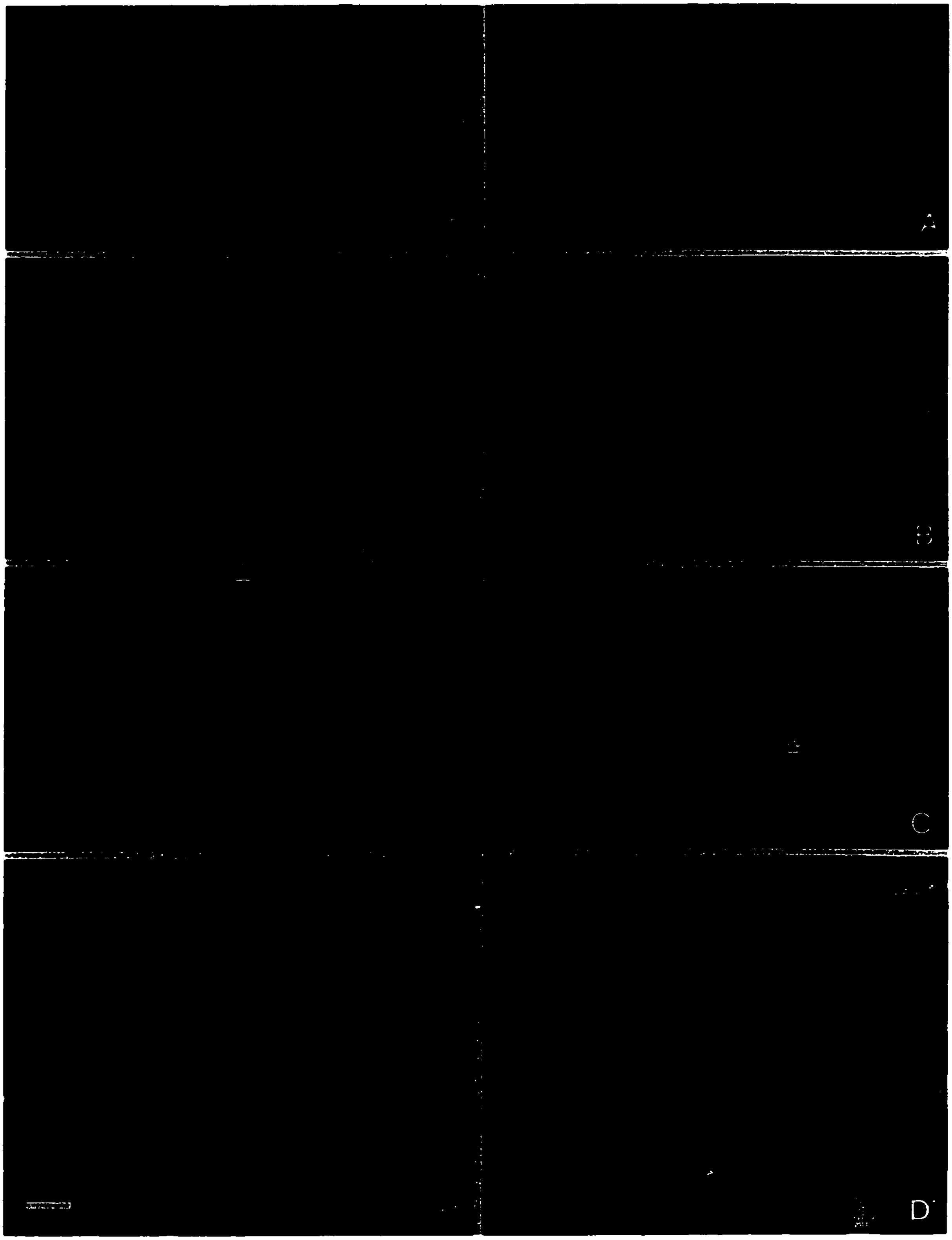

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
4
6
8
10
12
Day of neuronal differentiation
▨ P19
■ P19 2cmyc

with neuronally differentiating cultures of P19 cells, which results in differences in the accumulation pattern of proteins like MAP2. However, MAP2 protein levels were generally greater in the MAP2cmyc-transfected cell line when compared with protein levels obtained using control P19 cells (Fig. 17).

These results also show the stability of the Tα1-MAP2cmyc clone. After thawing and refreezing the clone several times, protein extracts from neuronally-induced cultures of these subclones were obtained. Western blotting and ELISA showed reproducible MAP2 and MAP2cmyc expression patterns with these protein extracts. These results were also confirmed by immunofluorescence microscopy.

To determine the cell type specificity of MAP2cmyc expression, subcellular distribution of the MAP2cmyc protein, and effects on cell morphology, a series of double labelling immunofluorescence experiments were carried out. Figure 18 is immunofluorescence microscopy of an RA-induced, neuronal timecourse of a Tα1-MAP2cmyc culture. Anti-MAP2cmyc staining was not detected in undifferentiated Tα1-MAP2cmyc cells (Fig. 18 A') and the microtubules, detected using an anti-α-tubulin antibody, appeared to be in a normal, MTOC-based arrangement (Fig. 18 A). Mitotic cells were also observed in these undifferentiated, transfected cultures. Labelling for the neuron-specific tubulin isotype, ß-III tubulin, was first observed 2 days after RA addition but no MAP2cmyc staining was apparent at that time (data not shown). Anti-myc staining was first detected three days

Figure 18. Double immunofluorescence staining of MAP2cmyc and ßIII-tubulin in neuronally-induced P19 cells, stably-transfected with Tα12cmyc and prepared by the simultaneous fixation/extraction method. A stained for α-tubulin using YOL1/34, B-D for neuron-specific ßIII-tubulin, and A′-D′ for the myc-epitope tag using 9E10. A-A′ are undifferentiated cells, B-B′ are Day 3 cells, C-C′ are Day 4 cells showing myc labelling in cell bodies (arrowhead) and into developing neurites (arrow), and D-D′ are Day 8 cells with MAP2cmyc labelling the length of a neurite (arrow). Bar=20μm



A
B
C
D

after RA induction, though most ß-III tubulin positive cells were not labelled with the anti-myc antibody (Fig. 18 B,B'). By day 4, neurons were more abundant in the culture and the length and number of neurites had increased. ß-III tubulin staining was more widespread and intense in these day 4 neurons and many also labelled for MAP2cmyc (Fig. 18 C,C'). As neuronal differentiation proceeded, ß-III tubulin staining increased and the number of myc positive neurons increased (Fig. 18 D,D').

At all days of differentiation, MAP2cmyc staining was most prominent in the cell bodies of developing neurons but was frequently found extending into one or more neurites (Fig. 18 C,C' and D,D'). The majority of myc labelled cells were located in cell aggregates where cell bodies and some neurites were intensely labelled by the anti-myc antibody. This localized staining pattern in aggregates was observed at the onset of MAP2cmyc expression (Fig. 18 C') and at later days of differentiation (Fig. 18 D'). MAP2cmyc-expressing neurons appeared morphologically indistinguishable from neurons not expressing the transgene.

MAP2 expression in neuronal cultures of the Tα1-MAP2cmyc clone was also characterized by double immunofluorescence microscopy and compared to untransfected P19 cultures. MAP2 was not detected in day 0 cultures of either cell line (Figs. 19 A and 20 A'). The MTs of Day 0 cells were brightly labelled with an anti-α-tubulin antibody and showed a normal, centrosome-based, radial pattern for both untransfected (Fig. 20 A) and transfected

Figure 19. Double immunofluorescence staining of MAP2 and MAP2cmyc in neuronally-induced P19 cells, stably-transfected with Tα12cmyc and prepared by the simultaneous fixation/extraction method. A-D stained for MAP2 using the anti-MAP2 polyclonal antibody, A′-D′ for the myc-epitope tag using 9E10, and A′′-D′′ for DNA. A-A′′ are undifferentiated cells, B′-B′′ are Day 3 cells, C-C′′ are Day 4 cells, and D-D′′ are Day 8 cells. Bar=10μm

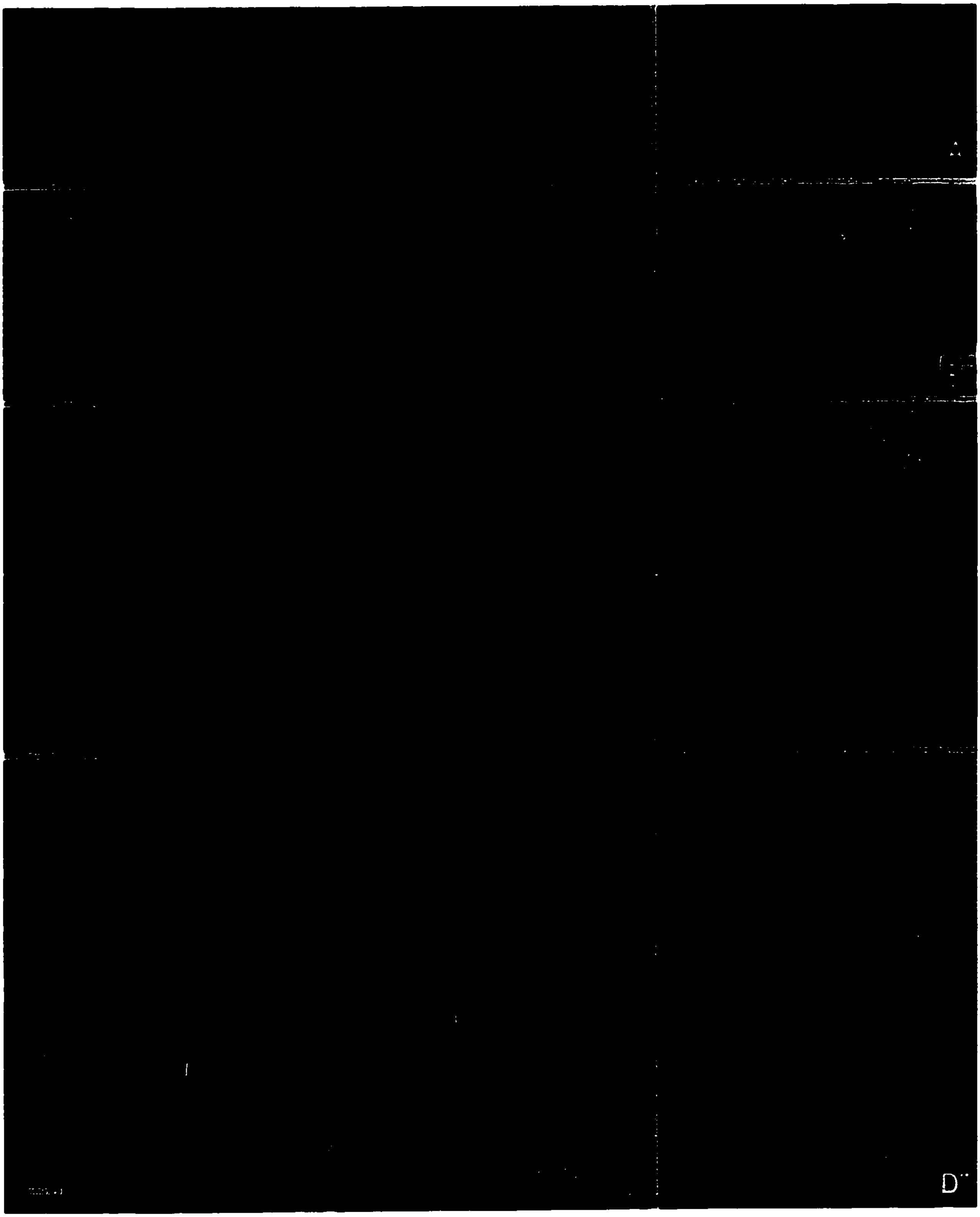
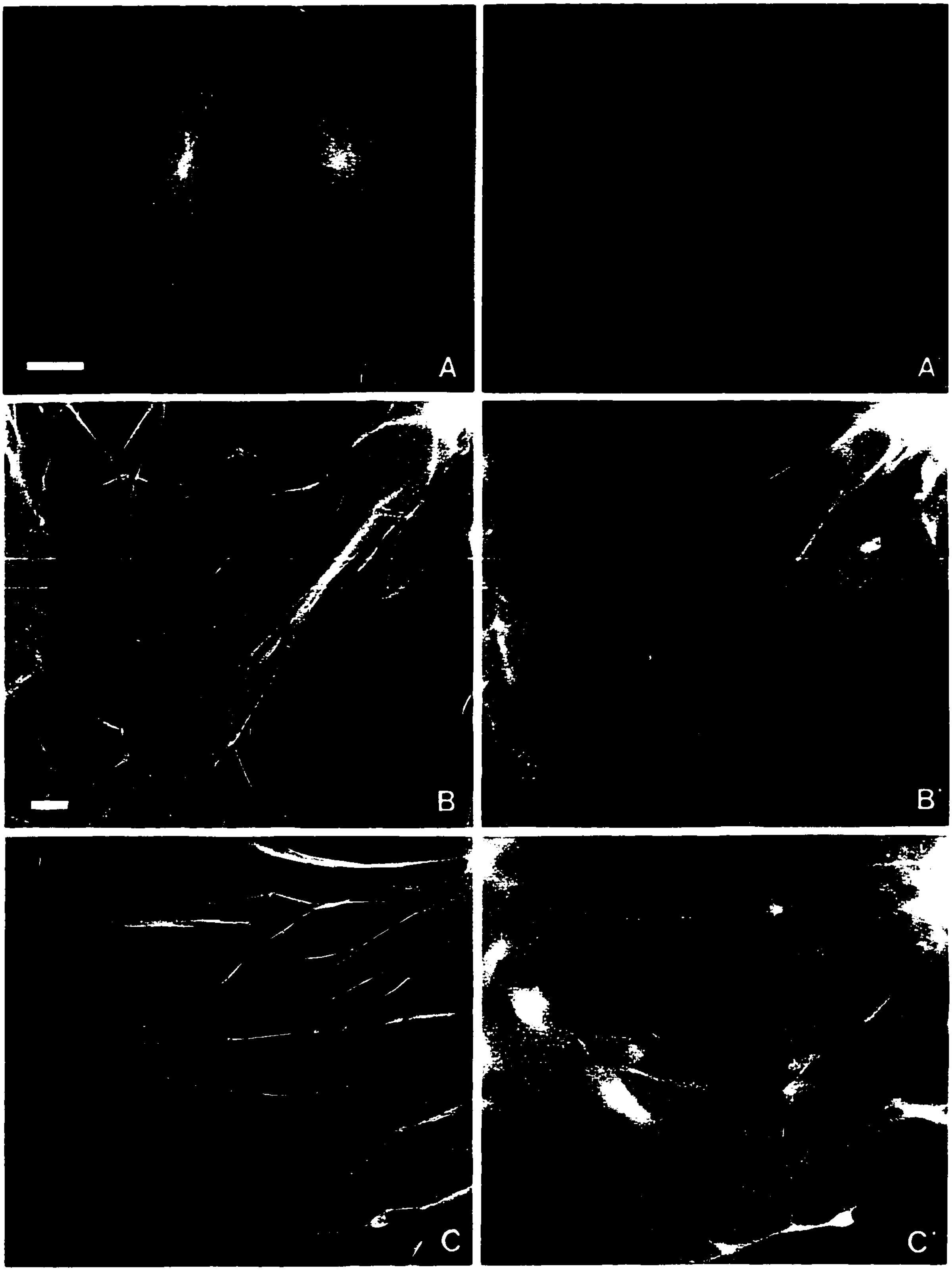

A
D''

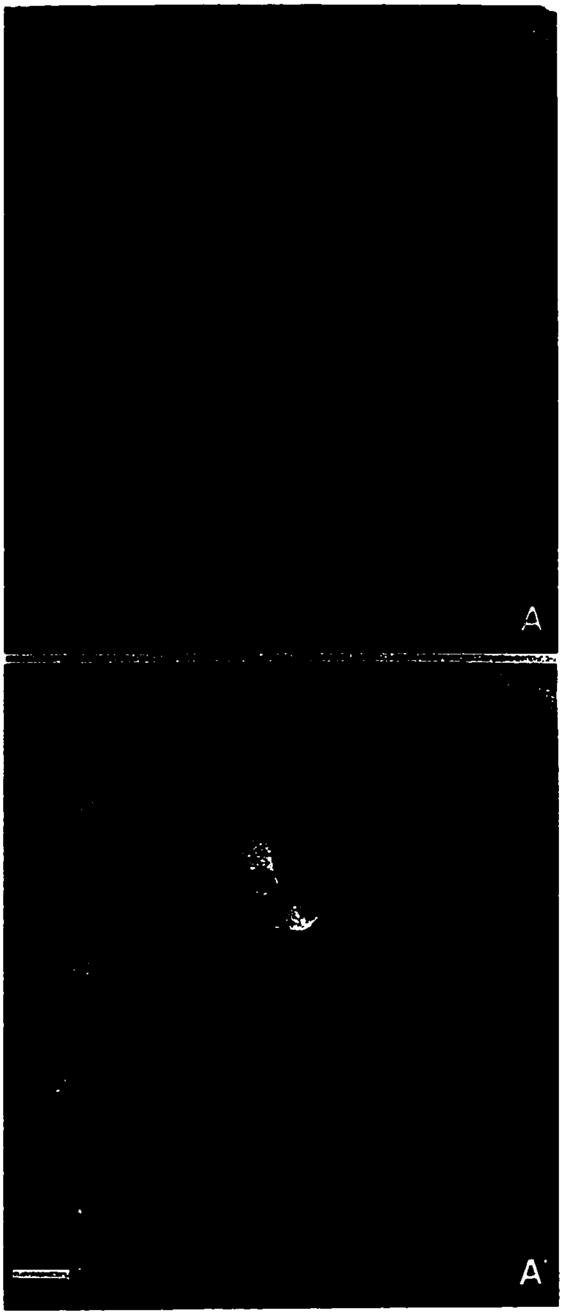
Figure 20. Double immunofluorescence staining of neuronally-induced P19 cells prepared by the simultaneous fixation/extraction method. A-C stained for α-tubulin using YOL1/34 and A′-C′ for MAP2 using HM-2. A-A′ are undifferentiated cells, B-B′ are Day 4 cells, and C-C′ are Day 8 cells. Bars=10μm


A
A'
B
B'
C
C'

(data not shown) cells. No anti-myc staining was detected in Day 0 cells for the stably-transfected cell line (Fig. 19 A′). MAP2 was first detected 2 days after the addition of RA (Day 2) in both control and MAP2cmyc-transfected cultures, confirming the Western blotting results (results not shown). MAP2cmyc labelling was absent in Day 2 cells of transfected cultures (data not shown). Labelling for MAP2 increased in day 3 cultures and a few myc positive cells were observed (Fig. 19 B, B′) though the majority of MAP2 positive neurons were not stained with the anti-myc antibody. As neuronal differentiation proceeded, MAP2 labelling became more extensive in both cell cultures (Figs. 19 C and 20 B′). The number of neurons staining with the anti-myc antibody increased in transfected cultures (Fig. 19 C′). By day 8 (Fig. 19 D′), myc-labelled neurons were abundant, particularly in cell aggregates. The number of MAP2 positive neurons and the intensity of MAP2 staining was greatest for both cell cultures by these later days of differentiation (Figs. 19 D and 20 C′). Figure 21 (A, A′) is a typical field of a day 8, Tα1-MAP2cmyc culture at lower magnification and clearly demonstrates the increase in myc-positive neurons at later days of differentiation. Labelling with MAP2 (Fig. 21 A) reveals a network of neurons, many of which also stained positive for MAP2cmyc (Fig. 21 A′).

To verify that MAP2cmyc expression was restricted to neurons, day 10 transfected cultures were double-labelled with the anti-myc antibody and an antibody to glial fibrillary acidic

Figure 21. Low magnification, double immunofluorescence staining of neuronally-induced P19 cells, stably-transfected with Tα12cmyc and prepared by the simultaneous fixation/extraction method. Cells were labelled for MAP2 using the anti-MAP2 polyclonal antibody (A) and the myc-epitope tag using 9E10 (A′) eight days following neuronal induction (Day 8). Bar=20μm


A
A'

protein (GFAP), which stains glial cells. GFAP and MAP2cmyc staining were mutually exclusive supporting the neuronal specificity of the Tα1 α-tubulin promoter (Fig. 22).

As an additional test of the neuron-specific nature of this promoter, the permanently transfected MAP2cmyc cell line was treated with DMSO to induce muscle differentiation and examined by immunofluorescence microscopy (Fig. 23). Because cells had to be prepared using two different methods in order to preserve the appropriate cytoskeletal protein and because both antibodies used are monoclonals, double-labelling with these two antibodies could not be performed. Undifferentiated and day 11 cells were, therefore, fixed and single-labelled with the anti-myc antibody and an antibody to muscle-specific myosin (MF20). DMSO-treated cells were also labelled with antibodies to ß-III tubulin and MAP2 to verify that neuronal differentiation was only rarely observed under these culturing conditions (results not shown). Neither protein was expressed in the undifferentiated Tα1-MAP2cmyc cell (Fig. 23 A,B). Several coverslips containing DMSO-treated cells were stained for MAP2cmyc and no myc labelling was detected (Fig. 23 C). Cells from the same DMSO treatment were labelled with the anti-myosin antibody and cells staining with the antibody were observed (Fig. 23 D).

Figure 22. Double immunofluorescence staining of neuron-specific MAP2cmyc expression in P19 cells, stably-transfected with Tα12cmyc and prepared by the simultaneous fixation/extraction method. Cells were stained for the myc-epitope tag using 9E10 (A) and glial fibrillary acidic protein (GFAP) (A′) ten days after neuronal induction. Bar=20μm

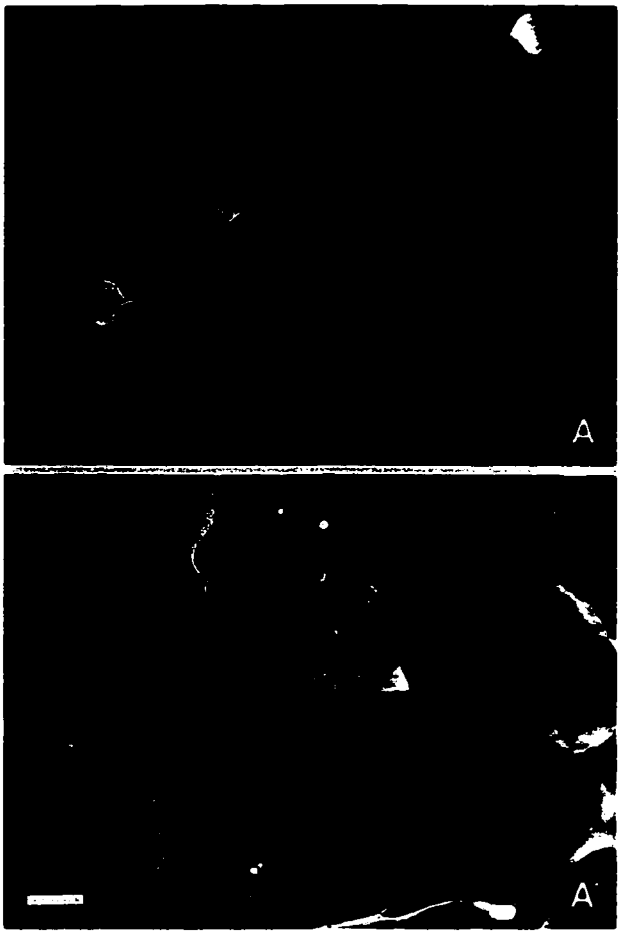
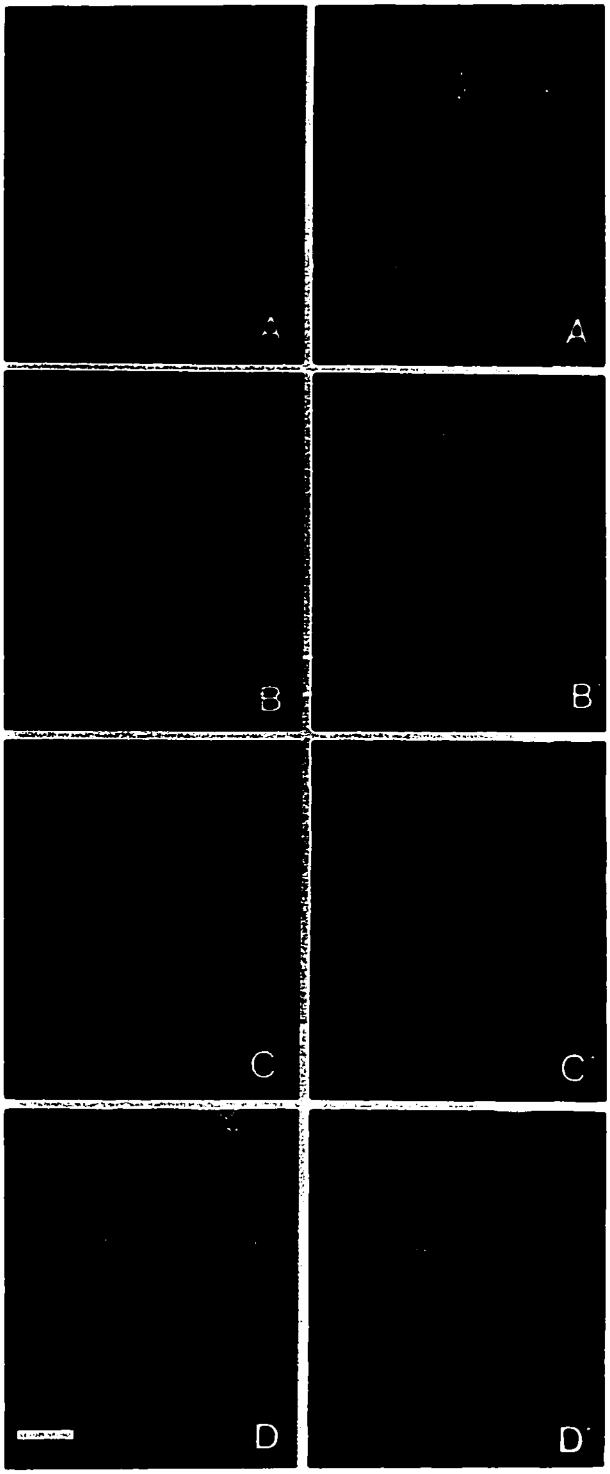

A
A'

Figure 23. Single immunofluorescence staining of DMSO-treated P19 cells, stably-transfected with Tα12cmyc. Cells prepared using the precipitative fixation method were labelled for MAP2cmyc using 9E10 (A,C) and cells prepared by the methanol fixation procedure were labelled for muscle-specific myosin using MF20 (B,D). Hoechst staining (A′-D′) was performed to visualize unlabelled cells. A-B′ are undifferentiated cells and C-D′ are cells treated with DMSO for 11 days. Bar=20μm


A
A'
B
B'
C
C'
D
D'

DISCUSSION

MICROTUBULE ORGANIZATION IN MAP2C AND HMW-MAP2 TRANSIENTLY TRANFECTED P19 CELLS

One approach taken when studying protein function is to express the protein of interest in cells in which it is not normally found. The resulting cellular phenotypes may indicate possible roles for the protein *in vivo*. In eukaryotic cells, this is accomplished by either directly microinjecting the protein, or transfecting its gene into cells so that the cells express the protein. For this comparative study on MAP2c and HMW-MAP2 function, the latter technique was chosen.

The transfection approach involves inserting the gene of interest into an appropriate expression vector and introducing it into cultured cells. The P19 EC cell line has been demonstrated to be a good candidate cell line for transfection studies (McBurney, 1993; MacPherson and McBurney, 1995) and was chosen for this investigation. The mouse phosphoglycerate kinase (PGK-1) promoter has been used for efficient, constitutive expression of a variety of transgenes in P19 cells (McBurney, 1993). In our laboratory, the PGK-1 promoter was used, previously, to effectively drive βIII-tubulin expression in transiently transfected P19 cells (Laferrière and Brown, 1994). Therefore, the PGK-1 promoter seemed to be a good candidate for driving constitutive expression of MAP2c and HMW-MAP2 in undifferentiated P19 cells.

MAP2c and HMW-MAP2 were independently transfected into P19 cells and the same alterations of MT organization were seen with both proteins (refer to Figs. 5 and 6). At least 50 percent of transfected cells displayed unaltered, radial MT arrays while others exhibited varying degrees of MT bundling. MT reorganization into densely packed MT bundles has been previously demonstrated in other cell types transfected with constructs encoding HMW-MAP2 (Lewis *et al.*, 1989), MAP2c (Takemura *et al.*, 1992; Umeyama *et al.*, 1993; Weisshaar *et al.*, 1992), MAP2d (Ferhat *et al.*, 1996), and the related MAP, tau (Kanai *et al.*, 1989; Knops *et al.*, 1991).

In this study, MAP2c- and HMW-MAP2-expressing cells that retained the normal MT arrangement appeared to stain less intensely for MAP2 compared to cells possessing MT bundles. The degree of MT bundling was, in general, greatest in cells that were also strongly labelled for MAP2 (refer to Figs. 5 and 6). Similar results were obtained by Lewis *et al.* (1989) with HMW-MAP2, and Weisshaar *et al.* (1992) with MAP2c. This suggests that greater MAP2 expression results in increased MT bundling and that a threshold level of MAP2 (MAP2c or HMW-MAP2) is required before MTs will associate to form bundles. Under this threshold limit, MTs are decorated with the protein but retain their normal, radial arrangement. Once the amount of MAP2 surpasses this limit, MTs begin to interact and form bundles. Lewis *et al.* (1989) have previously shown a qualitative correlation between MT bundling and the level of MAP2 expression, judged by fluorescence intensity.

In their study, Weisshaar *et al.* (1992) used a rapid freezing cell fixation method to prepare MAP2c-transfected cells for staining and found that all MAP2c was bound to MTs. In contrast, MAP2c expressed in transiently transfected P19 cells was detected in the cytoplasm when a precipitative method was used to prepare cells. In this method, soluble proteins are precipitated in the cytoplasm and are not removed by the subsequent cell permeabilization with detergent. Conversely, transfected cells that had been simultaneously fixed and extracted showed MAP2c only bound to MTs. The number of cells with detectable levels of MAP2c was also greater in cultures that had been prepared by the precipitative fixation method. This result is due to better preservation and detection of small amounts of soluble MAP2c in low level MAP2c-expressing cells by this fixation method. The simultaneous fixation/extraction protocol removes the small of amount of MAP2c in these low level expressors, making them undetectable by microscopy. The results of Weisshaar *et al* (1992) are similar to those obtained by the simultaneous fixation/extraction method suggesting that soluble proteins are removed by both of these methods.

Western blot results showed at least two MAP2c bands in MAP2c-transfected cell extracts (refer to Fig. 7). One band, migrating faster than MAP2c from crude brain extract, was probably unmodified MAP2c. A posttranslational modification is likely to have occurred on transfected MAP2c, resulting in the appearance of the second band. MAP2c is known to be phosphorylated *in vivo* (Tsuyama *et al.* 1987; Brugg and Matus, 1991) and the heterogeneity of MAP2c in brain extracts is removed after dephoshorylation (Crandall and Fischer, 1989). This second band, therefore, is probably due to the phosphorylation of transfected MAP2c since it migrated with MAP2c from crude brain extract.

MICROTUBULE STABILITY IN MAP2C AND HMW-MAP2 TRANSIENTLY TRANSFECTED P19 CELLS

MAP2 proteins have been previously demonstrated to stabilize and bundle MTs when expressed in cells normally devoid of MAP2 (Ferhat *et al.*, 1996 Lewis *et al.*, 1989; Takemura *et al.*, 1992; Weisshaar *et al.*, 1992). When transiently transfected into P19 cells, MAP2c and HMW-MAP2 stabilized MTs against colchicine-induced depolymerization, supporting these earlier reports. Stable MT bundles were also enriched in acetylated α-tubulin, an indicator of increased MT stability. The unbundled MTs in transfected P19 cells were less resistant to colchicine depolymerization and did not label for acetylated tubulin. Takemura *et al.* (1992) showed similar results when MAP2c and tau were transiently transfected into fibroblast cells.

The increase in MT stability of MAP2c- and HMW-MAP2-transfected P19 cells may explain why groups of transfected cells were rarely seen. When observed, these cells had normal MT arrays and expressed the transgene at low levels. Since cells require the more dynamic MTs of the spindle apparatus for proper mitosis and cell division, an inappropriate increase in MT stability would likely impede these processes. This also explains the infrequent occurrence of transfected, mitotic cells in these cultures. In contrast, stable transfections with the related protein tau have been successfully conducted in fibroblast cell lines (Kanai *et al.*, 1989; Lo *et al.*, 1993) and Chinese Hamster Ovary cells (Barlow *et al.*, 1994). However, tau has been shown to stabilize MTs to a lesser degree than MAP2c (Takemura *et al.*, 1992), allowing for normal mitotic spindle function and the proliferation of these stably transfected cell lines.

To date, no attempt to compare the MT stabilizing abilities of MAP2c and HMW-MAP2 has been made. In this study, the MT stabilizing abilities of MAP2c and HMW-MAP2 were determined by measuring the percentage of transfected P19 cells possessing stable MT bundles after various colchicine treatment times. Stable MT bundles were observed after all colchicine treatments and the percentage of stable MT bundles decreased as colchicine exposure increased for both MAP2c and HMW-MAP2 transfected cells (refer to Fig. 12). The slopes of the curves were used to compare MT stability conferred by MAP2c and HMW-MAP2. By ANCOVA, the slopes were not significantly different indicating that the MT stabilizing abilities of MAP2c and HMW-MAP2 are the same.

Alternatively, MTs may be better stabilized by HMW-MAP2 but MAP2 expression on a per cell basis may be greater in MAP2c-transfected cells than in HMW-MAP2-transfected cells, masking a true difference in MT stability. As discussed earlier, MT bundling and stability were greater in cells expressing high amounts of either MAP2 protein. Western blotting showed that MAP2c expression was much higher than HMW-MAP2 expression in transiently transfected cell populations. This difference in population expression levels could be partly due to expression level differences in individual cells. If individual MAP2c-transfected cells were, in fact, expressing more protein on average than HMW-MAP2-transfected cells, then MT bundle stability would appear similar in both populations of transfected cells.

STABLE EXPRESSION OF MYC-TAGGED MAP2C IN NEURONALLY-INDUCED P19 CELLS

The transient transfection of neuronal proteins like MAP2 and tau in non-neuronal cell lines has provided insight into the *in vivo* functions of these proteins. However, the cellular environments of non-neuronal cells and neurons are different and could affect the behaviour of transgenic proteins. For this reason, the effect of neuronal protein expression in undifferentiated cells must be considered carefully.

The choice environment for examining neuronal protein function is inside neurons. Transient transfection studies on primary cultured neurons, using epitope-tagged forms of tau, MAP2c and HMW-MAP2, have been performed previously (Meichsner *et al.*, 1993; Kanai and Hirokawa, 1995). However, variations in plasmid copy number picked up and expressed in transiently transfected cells results in a range of observed biological effects. The stable transfection of neuronal precursor cells circumvents this problem since neurons are derived from a single clone and the number and location of gene copies are identical (ie. one transformation). Tau has been stably transfected into the nerve growth factor (NGF)-responsive PC12 cell line (Esmaeli-Azad *et al.*, 1994) to examine effects on neuronal differentiation. Tau overexpression resulted in increased MT mass and neuritic growth compared to control PC12 cells.

This study represents the first stable transfection with MAP2 in cultured cells. As discussed earlier, the lack of earlier reports is most likely due to the strong MT stabilizing effect of MAP2 on the mitotic spindle, which would prevent the proliferation of a constitutively MAP2-expressing cell line. PGK-driven, constitutive expression of myc-tagged MAP2c in neuronally-induced P19 cells resulted in the rapid decrease in detection of the transgene over time (refer to Fig. 14). Few myc positive cells were seen by the time ßIII-tubulin-stained neurons were first observed and these cells appeared to label weakly, or not at all, for neuron-specific ßIII-tubulin. MAP2c expression in undifferentiated cells did not induce early neuronal differentiation. The decrease in abundance of MAP2cmyc-expressing cells, as neuronal differentiation proceeded, indicates that MAP2c expression prior to neuronal commitment is detrimental to cell function and likely leads to cell death. Since proliferation is inhibited when differentiating cells are cultured in the defined neuronal medium (MacPherson and McBurney, 1995) and transfected cells were numerous before neuronal induction, it is unlikely that the decrease in abundance is a dilution effect.

Using the developmentally regulated, tissue-specific human cardiac actin (C^H-actin) promoter, Rudnicki et al. (1988, 1989) and Pari et al. (1991) were successful in driving appropriate developmental and muscle-specific expression of several genes in DMSO-induced P19 cells . The present study demonstrates that stable expression of the MT-stabilizing protein MAP2c in developing P19 neurons is better achieved using a developmentally-regulated, neuronal promoter.

The effect of MAP2c overexpression on neuronal morphogenesis was investigated using a P19 cell line stably-transfected with myc-tagged MAP2c (Tα1-MAP2cmyc). MAP2cmyc expression in this cell line was under the control of the neuron-specific Tα1 α-tubulin promoter. In the developing rat embryo, the Tα1 gene is expressed at high levels, and exclusively in differentiating neurons (Miller *et al.*, 1987). When used to drive nuclear-localized ß-galactosidase expression in transgenic mice, the transgene showed the same developmental and neuron-specific expression pattern as the endogenous Tα1 α-tubulin gene (Gloster *et al.*, 1994; reviewed in Miller *et al.*, 1996). The Tα1 promoter was also used to drive stable, neuron-specific ß-galactosidase expression in neuronally-induced P19 cells (Laferrière, 1995; Rogers *et al.*, 1995). Transgene expression also showed developmental regulation, with the ß-galactosidase protein being first detected three days following neuronal induction.

MAP2c expression, driven by the Tα1 promoter, was restricted to neurons of RA-induced P19 cultures, supporting the findings of Gloster *et al.* (1994), Laferrière (1995) and Rogers *et al.* (1995) (refer to Fig. 16). The non-specific binding of the anti-myc antibody to proteins in P19 WC extracts (refer to Fig. 15) had no influence on the immunofluorescence microscopy observations since no anti-myc staining was detected in Day 0 cells. It is unlikely, therefore, that this non-specific binding contributed to the anti-myc immunofluorescence staining seen in cultures differentiated for three days or longer. The neuronal specificity of this promoter was demonstrated in double-labelled Tα1-MAP2cmyc cultures showing mutually exclusive GFAP and MAP2cmyc staining (refer to Fig. 21). Furthermore, MAP2cmyc staining was not detected in myosin-expressing cells of DMSO-treated cultures (refer to Fig. 22).

Western blotting and ELISA revealed MAP2cmyc to have similar protein accumulation patterns as endogenous MAP2c in neuronally-induced cultures (refer to Figs. 15 and 16). However, by Western blotting, MAP2cmyc was not detected until three days after the addition of RA, one day later than the appearance of endogenous MAP2c. Immunofluorescence microscopy, at this stage, showed the beginning of neurite elaboration and extensive ßIII-tubulin staining (refer to Fig. 17). This result is consistent with the data obtained by Laferrière (1995) using Tα1 to drive ß-galactosidase expression in permanently transfected P19 cells. The Tα1 promoter is activated following the neuronal induction of P19 cells, one day after the appearance of ßIII-tubulin and endogenous MAP2c, and coincides temporally with neurite outgrowth.

Besides being detected one day later than endogenous MAP2c, MAP2cmyc protein levels also declined at the same time as the endogenous protein. One explanation for the decrease in MAP2c and MAP2cmyc could be a reduction in mRNA transcription from both the endogenous MAP2 and exogenous Tα1 promoters. MAP2c is associated with neuronal growth because of its characteristic abundance in the developing brain and its presence in growing neurons of the adult brain (Tucker *et al.*, 1988; Crandall and Fischer, 1989). Similarly, Tα1 α-tubulin mRNA expression is maintained at high levels during developmental growth of neurons, and decreases once target contact is established (Miller *et al.*, 1987). The association of both MAP2c and Tα1 α-tubulin expression with neuronal growth, and the subsequent decrease in expression following neuronal maturation, may also be reflected in P19 neuronal cultures.

The decrease in protein levels during the later days of neuronal differentiation was also observed for HMW-MAP2 in both transfected and untransfected cultures. Although the neuronal induction of P19 cells results in a population enriched with neurons, other neural cell types also develop in culture. The differentiation of glia, demonstrated by GFAP staining, occurred later in culture (days 8 to 10). Rapid glial cell differentiation would result in the production of glial-specific proteins which would effectively dilute out MAP2 and other neuronal proteins.

Subcellular localization of MAP2cmyc was also investigated. MAP2cmyc staining was most prominent in the cell bodies of neurons expressing the transgene but frequently extended into one or more neurites which suggests that MAP2c may be transported into, or translated in, all processes (refer to Fig. 17). This observation supports previous work on developing motor neurons and retinal ganglion cells, in which MAP2c was found in both the axons and dendrites of these cells (Tucker *et al.*, 1988; Albala *et al.*, 1995; Tucker and Matus, 1988). MAP2c transfection studies on cultured hippocampal neurons (Meichsner *et al.*, 1993) also demonstrate epitope-tagged-MAP2c labelling of all processes. Dendrites and the proximal region of axons were stained for tagged-MAP2c in transfected spinal cord neurons (Kanai and Hirokawa, 1995).

Other studies have reported MAP2c sorting to the dendrites and cell bodies in most regions of the developing brain (Bernhardt and Matus, 1984; Burgoyne and Cumming, 1984). Marsden *et al.* (1996) demonstrated MAP2c sorting to dendrites in the adult brain of transgenic mice. Hirokawa *et al.* (1996) showed biotinylated MAP2c and tau to be initially localized in both the axonal and dendritic compartments of cultured neurons, with MAP2c and tau persisting only in dendrites and axons, respectively.

A possible explanation for these differences in subcellular localization is that specialized classes of neurons may require specific distributions of MT stabilizing proteins, like MAP2c and tau, to maintain their different morphologies. For example, the exceptionally long axons of motor neurons may require the increased MT stability and stiffness provided by MAP2c.

Although all neurons derived from the original Tα1-MAP2cmyc clone should express the MAP2c transgene, only a subset of neurons labelled with the anti-myc antibody. Similarly, Bamji and Miller (1996) raised a line of transgenic mice where Tα1-driven nuclear-localized β-galactosidase expression was only observed in a subset of neurons in a given population. However, Laferrière (1995) generated a P19 cell line that stably expressed nuclear-localized β-galactosidase in the majority of neurons by later days of neuronal differentiation.

The most likely explanation, with regards to the MAP2cmyc and β-galactosidase stable P19 clones, may lie with the different subcellular distributions of the two proteins. MAP2cmyc was distributed throughout the cytoplasm, while β-galactosidase was targeted to the nuclei of transfected neurons. The detection of transgenic β-galactosidase protein may have been better than that of MAP2cmyc due to its concentration in the nuclei of neurons, resulting in a bright, easily visualized signal by immunofluorescence microscopy.

Another goal of this study was to investigate the effect of MAP2c overexpression on the general morphology of developing neurons. Despite the increase in MAP2c expression, revealed by Western blotting and ELISA, these neurons appeared morphologically indistinguishable from control, untransfected P19 neurons. This result is surprising since PC12 cells, stably transfected with the related MAP, tau, showed increased MT mass and neuritic growth (Esmaeli-Azad *et al.*, 1994). Further, reduced MAP2 expression resulted in the inhibition of neuritic growth with cultured rat cortical neurons (Sharma *et al.*, 1994) and cerebellar macroneurons (Caceres *et al.*, 1992). Similarly, Dinsmore and Solomon (1991) conducted antisense MAP2 experiments in P19 cells and found that constitutive expression of MAP2 antisense RNA resulted in reduced levels of MAP2 protein and inhibition of neurite extension.

Unlike these previous investigations, the present study suggested that increased MAP2c expression in P19 neurons had no obvious effect on neuronal morphology. This may have been because only a portion of the MAP2cmyc protein bound to MTs. Although transiently transfected P19 cells prepared by the simultaneous fixation/extraction method showed MAP2cmyc protein associated with the MTs of these cells, transfected cells prepared using the precipitative method revealed intense MAP2 cytoplasmic staining. This result illustrates that not all transfected MAP2 protein was associated with MTs.

However, Marsden *et al*. (1996) showed that MAP2c expression in the adult mouse brain resulted in no detectable difference in the neuronal morphology of transfected animals, which supports the observations made with this MAP2cmyc permanently-transfected cell line. In addition, Viereck *et al*. (1989) showed that neurons from the adult olfactory system express MAP2c, continue to undergo morphogenic changes during adult life, and do not appear to differ morphologically from neurons of other regions in the adult brain. These results suggest that, while MAP2c has some function in the morphogenesis of growing neurons, other neuronal proteins may play more significant roles in this process.

FUTURE WORK

The Tα1 α-tubulin promoter demonstrated neuron-specific expression, and appropriate developmental regulation in P19 cells stably transfected with MAP2cmyc, making it a powerful tool for investigating protein function during neuronal differentiation. For example, the Tα1 α-tubulin promoter could be used to drive overexpression of other MAPs (neuronal or not) and tubulin isotypes in differentiating P19 neurons.

A HMW-MAP2 stably-transfected P19 cell line could be generated, allowing for a comparative study of MAP2c and HMW-MAP2 function in differentiating neurons. Ultimately, the effects of MAP2c and HMW-MAP2 overexpression on MT stability and neuronal morphology could be compared. The Tα1 promoter could also be employed to drive the antisense RNA expression of specific proteins, including MAP2, in differentiating P19 neurons. The effects of underexpression on neuronal differentiation could then be examined for these proteins.

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IMAGE EVALUATION
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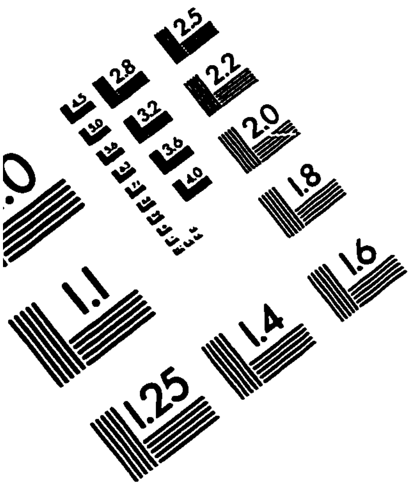

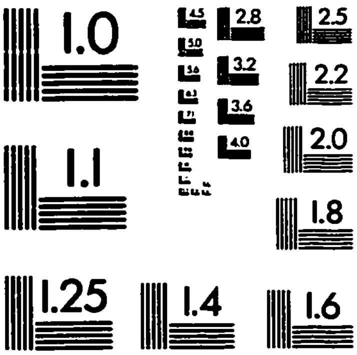

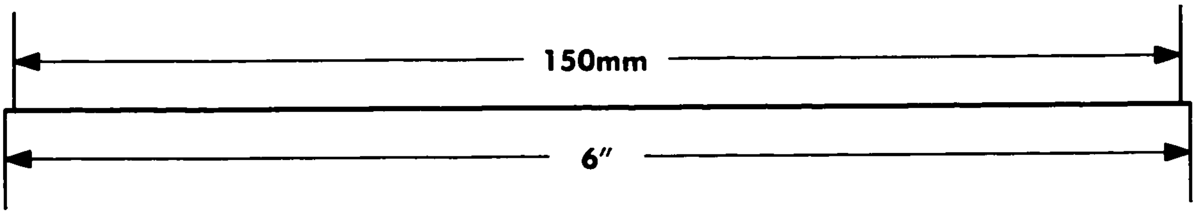

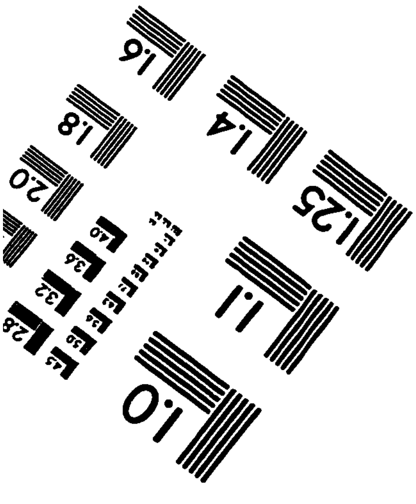

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