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EXPRESSION AND POSTTRANSLATIONAL MODIFICATION
OF CLASS III β -TUBULIN DURING NEURONAL
DIFFERENTIATION OF P19 EMBRYONAL CARCINOMA CELLS

by

Nicole B. Laferrière

A thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfilment of the requirements
for the degree of
Doctor of Philosophy
Ottawa-Carleton Institute of Biology



Nicole Laferriere, Ottawa, Canada, 1996



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Nicole B. Laferrière

To Rowan and Mackenzie,
with love

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ABBREVIATIONS

α -MEM	α -modified Eagle's minimal essential medium
BES	N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid
cdNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
cs	coverslip
CY3	indocarbocyanine
DTAF	dichlorotriazinylamino fluorescein
ECL	enhanced chemiluminescence
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DTAF	dichlorotriazinylaminofluorescein
EC	embryonal carcinoma
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol bis(β -aminoethylether) N,N,N',N'-tetra-acetic acid
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
IEF	isoelectric focusing
IPG	immobilized pH gradient
<i>lac-z</i>	<i>E. coli</i> gene encoding enzyme β -galactosidase
MAP	microtubule-associated protein
MEM	modified Eagle's medium
MES	N-morpholinoethanesulfonic acid
Mt	microtubule
MTOC	microtubule organizing center

mRNA	messenger ribonucleic acid
MSB	microtubule stabilizing buffer
PBS	phosphate buffered saline
PEM	piperazine-N,N,-bis(2-ethanesulfonic acid) / EGTA/MgCl ₂ buffer
<i>Pgk-1</i>	phosphoglycerate kinase
RA	retinoic acid
RT	room temperature
SDS	sodium dodecylsulphate
<i>Tα1</i>	neuronal rat α -tubulin
TRIS	tris(hydroxymethane) aminomethane
UTR	untranslated region
X-GAL	5-bromo-4-chloro-3-indolyl- β -galactopyranoside

ABSTRACT

The functional significance of Class III β -tubulin during neurogenesis has been investigated. A combination of northern blotting, immunofluorescence microscopy, enzyme-linked immunosorbent assay, and isoelectric focusing was used to characterize the expression of β -III tubulin in P19 embryonal carcinoma cells induced to differentiate along a neuronal pathway by retinoic acid. Following 48 hours differentiation, β -III tubulin mRNA is evident and β -III tubulin appears in the mitotic spindle of neuronal precursors. Neurite outgrowth is obvious by day 3, and β -III tubulin and mRNA levels increase concurrently until approximately day 8, when β -III mRNA levels begin to decrease while protein levels remain high. In addition, increasingly acidic β -III isoforms appear with days of differentiation. The accumulation of these isoelectric variants occurs concomitant with a temporal increase in the incorporation of β -III tubulin into colchicine-stable microtubules.

To investigate the relationship between polymerization and posttranslational modification of β -III tubulin, undifferentiated P19 cells and P19 neurons were treated with the alkaloid taxol, which promotes polymerization of stable microtubules.

P19 cells were grown on coverslips and then treated with taxol at concentrations from 10^{-6} M to 10^{-9} M for 24 hours. The microtubule cytoskeleton was examined after double-immunofluorescence labelling with a monoclonal antibody to α -tubulin (YOL 1/34) and a monoclonal neuron-specific β -III tubulin antibody (TuJ1). Treatment of undifferentiated P19 cells with

concentrations of taxol greater than 4×10^{-8} M causes microtubule bundling and multiple aster formation and promotes polymerization of the low levels of β -III tubulin found in these cells. In neurons, at 2×10^{-8} M taxol, bundling of microtubules at the base of the neurite is apparent. At taxol concentrations greater than 10^{-7} M enhanced assembly of β -III tubulin is noted, although long neurites are not observed. Using isoelectric focusing followed by western blotting, an additional isoform of β -III tubulin is detected following treatment with 10^{-6} M taxol. These results indicate that taxol treatment alters the normal subcellular sorting of tubulin isotypes, promotes the polymerization and posttranslational modification of β -III tubulin, and interferes with neurite outgrowth.

The coding sequence of mouse β -III tubulin was transiently expressed in undifferentiated P19 cells and its assembly and resistance to colchicine-depolymerization examined by immunofluorescence microscopy and isoelectric focusing. In these cells, β -III tubulin is detected in typical, centrosome-based microtubule arrays as well as in mitotic spindles and midbodies. Two β -III tubulin isoforms are detected on isoelectric focusing immunoblots. Microtubule disassembly by colchicine appears identical in the transfected and adjacent non-transfected cells. These results reveal that Class III β -tubulin is assembly competent and can undergo at least one posttranslational modification in non-neuronal cells. This expression, however, is insufficient to confer colchicine-stability to the microtubules in undifferentiated P19 cells.

Several posttranslational modifications of β -III tubulin have been identified, including phosphorylation of Ser⁴⁴⁴ and/or Tyr⁴³⁷, and glutamylation of Glu⁴³⁸. Of these, phosphorylation of Ser⁴⁴⁴ is unique to β -III tubulin in brain. To investigate the role of Ser⁴⁴⁴ phosphorylation of β -III tubulin, site-directed mutagenesis was used to generate two mouse β -III tubulin mutants. In one, Ser⁴⁴⁴ was replaced by Ala⁴⁴⁴ to provide a β -III tubulin molecule which could not undergo phosphorylation at that site. In the second mutant, Ser⁴⁴⁴ was replaced by Glu⁴⁴⁴ to mimic a constitutively phosphorylated protein. When β -III tubulin Ser⁴⁴⁴ mutant expression vectors are transiently expressed in P19 cells, examination by immunofluorescence microscopy reveals that both mutant β -III tubulins yield labelling patterns which are similar to the wild type β -III tubulin. Treatment with colchicine for 45 min results in disassembly of microtubules similar to that noted in non-transfected cells and those cells transfected with wild-type β -III tubulin. Thus, neither β -III tubulin mutation confers resistance to 45 min colchicine-induced depolymerization in non-neuronal cells.

The results of this study correlate for the first time, the appearance of developmentally-regulated posttranslational modifications of β -III tubulin with increased incorporation of β -III into stable microtubule arrays. In spite of its restricted pattern of expression, β -III tubulin can assemble and undergo at least one posttranslational modification in non-neuronal cells. This modification, however is insufficient to confer colchicine-stability to these microtubules. β -III tubulin Ser⁴⁴⁴ mutants did

not appear to modulate the colchicine-stability of microtubules containing β -III tubulin. These results implicate the more acidic posttranslational modifications in the increased colchicine-stability of microtubules containing β -III tubulin noted during development of P19 neurons.

The cistern contains: the fountain overflows

William Blake

FOREWORD

The dynamic network of filamentous structures found in the cytoplasm of eukaryotic cells is known as the cytoskeleton. Three types of cytoskeletal structures have been identified: microtubules (24 nm diameter), intermediate filaments (7 to 10 nm diameter), and actin microfilaments (7 nm diameter). The subunits of these filaments are proteins which self-assemble in noncovalent associations to form long polymers. Each family of fibres exhibits distinctive biochemistry, cellular distribution, and function, and each contributes in a specialized way to the generation and maintenance of cell shape. It is important when considering any group of cytoskeletal fibres, to appreciate the complexity of their interactions with each other, and with associated proteins, in the formation and function of the cytoskeleton. This thesis examines only one group of cytoskeletal proteins, microtubules.

Microtubules (Mts) are ubiquitous fibres found in the cytoplasm of eukaryotic cells. In addition to providing internal structural support and aiding in the spatial organization of various cytoplasmic organelles, they are involved in ciliary- and flagellar-dependent movement. They serve as an intracellular substrate for the transport of small vesicles and various organelles such as the Golgi and endoplasmic reticulum. They form the mitotic and meiotic spindle and segregate replicated chromosomes during cell division. Mts play a significant role in the morphogenesis associated with the differentiation of specialized cells (Inoué, 1982; reviewed by Kirschner and Mitchison, 1986).

With few exceptions, the ultrastructural appearance of Mts is identical, regardless of function or localization. How then, are these apparently indistinguishable structures organized to fulfil such diverse cellular functions? This question has compelled researchers to examine the molecular genetics and biochemistry of tubulin, the structural subunit of all Mts. Other proteins, found to be associated with these fibrillar structures, have been investigated in a similar fashion.

Such studies have shown that these apparently similar fibres exhibit a complex biochemical profile. Variations in Mt dynamics, tubulin expression and posttranslational modification, and patterns of Mt binding with various microtubule-associated proteins (MAPs), have been observed between species, tissue types, and even within single cells (reviewed by Sullivan, 1988).

Neurons are highly specialized cells which exhibit a characteristic polarized cellular morphology as a result of unique cytoskeletal arrangements. A typical neuron consists of a cell body (perikaryon) from which extend long neurites (one axon and none or as many as several arborized dendrites). Mts are essential for the development and maintenance of this unique cellular morphology (reviewed by Joshi and Baas, 1993). They are involved in the mitotic division of differentiating neuronal precursors, neuron migration, neurite outgrowth, and intracellular transport of organelles and proteins. They play a role in neuronal plasticity and possibly long term potentiation involving memory and learning (reviewed by Burgoyne, 1990). As a result, the mechanisms involved in the regulation, establishment and maturation of this elaborate

cytoskeletal network have generated considerable interest.

The introduction is a brief review of literature examining Mt structure and organisation in neurons, with emphasis on Class III β -tubulin during neurogenesis. It is followed by my hypothesis and a brief explanation of the rationale supporting my experimental approach.

INTRODUCTION

MICROTUBULE STRUCTURE

The structural protein subunit of all microtubules (Mts) is, a 100 kD protein heterodimer, consisting of one 50 kD alpha- and one 50 kD beta-polypeptide. Tubulin molecules polymerize in a head-to-tail fashion forming linear units known as protofilaments. Thirteen protofilaments form lateral associations to create a Mt, an apparently hollow tube with a diameter of 24 nm. Protofilaments are assembled with α - and β -tubulin subunits alternating, resulting in a polarized structure, with α -tubulin exposed at one end of the Mt and β -tubulin exposed at the other. Assembly and disassembly of tubulin dimers takes place preferentially at one end of the Mt designated the plus (+) end, with little assembly taking place at the other minus (-) end (Binder et al., 1975) which is generally embedded in the microtubule-organizing centre.

DYNAMIC INSTABILITY

Mts present in many cell types are highly dynamic, and exhibit an average half-life of approximately 5 minutes, while maintaining a constant Mt mass (Shulze and Kirschner, 1986). The coexistence of shrinking and growing Mts is thought to be the result of differences in the (+) or the fast growing end of the Mts.

Each α - and β -polypeptide in the tubulin heterodimer contains one bound guanosine triphosphate (GTP) molecule. At some point after the incorporation of tubulin into the growing

Mt, the β -tubulin GTP molecule is hydrolysed to guanosine diphosphate (GDP) (David-Pfeuty et al., 1977), altering the conformation of this tubulin subunit and thereby lowering the affinity for neighbouring subunits. The α -tubulin GTP is not hydrolysed in the assembly reaction (Spiegelman et al., 1977), and it probably plays a structural role (reviewed by Burns, 1991). As GDP-tubulin will dissociate more readily than GTP-tubulin, accumulation of GDP-bound tubulin renders a Mt unstable and results in rapid depolymerization (reviewed by Kirschner and Mitchison, 1986). If more free GTP-tubulin is available and binds to the growing end of the Mt before the GTP of earlier tubulin subunits has been hydrolysed, the Mt remains sufficiently stable for assembly to continue. In this way, both the concentration of free tubulin and the rate of GTP hydrolysis are important factors in the regulation of Mt growth and disassembly (Mitchison and Kirshner, 1984). Both the mechanical and structural properties of GTP-tubulin have been shown to differ from GDP-tubulin (Vale et al., 1994).

Differences in Mt dynamics have been operationally defined using a variety of experimental manipulations, such as the relative resistance of Mts to depolymerization induced by low temperature and drugs such as colchicine. Mt dynamics have also been examined using microinjection techniques where the rate of labelled tubulin subunit turnover has been measured directly.

The dynamic properties of Mts have been shown to vary between cell types (Pepperkok et al., 1990) and even between

specific locations within a single cell (Shulze and Kirschner, 1987). Postmitotic cells, such as neurons, establish a less dynamic population of Mts (Black and Greene, 1982; Lim et al. 1989; Falconer et al. 1989a, 1992). The mechanism(s) by which Mts are differentially stabilized is not yet known.

TUBULIN HETEROGENEITY IN HIGHER EUKARYOTES

Both α - and β -polypeptides constitute a multigene family of biochemically-distinguishable tubulin isotypes. The term "isotype" refers to a polypeptide sequence which varies by at least one amino acid substitution from other tubulins expressed within the same organism, and which shows conservation of similar sequence differences across species (Little and Seehaus, 1988). An additional isotype, γ -tubulin, has been identified more recently, and is primarily associated with the pericentriolar material of the centrosome. It is thought to form heterodimers with α - or β -tubulin, and is important for the nucleation of Mts in Mt organizing centres (Oakley and Oakley, 1989). It will not be discussed further in this thesis.

Most organisms express multiple α - and β -tubulin isotypes, with characteristic patterns of isotype expression noted within specific cell types. Within a single neuron, for example, several α - and β -tubulin genes are expressed (Gozes and Sweadner, 1981). In vertebrates, 6 α - and 6 β -tubulin isotypes have been highly conserved during evolution with the approximately 450 amino acid residues of α - and β -tubulin sequences exhibiting 36-42% homology. The amino acid sequence

homology within α - and β -tubulin isotypes is much higher. The protein sequence and pattern of expression of both α - and β -tubulin isotypes exhibits a high degree of conservation in the same cell types of different species (Sullivan and Cleveland, 1986; Little and Seehaus, 1988).

The high molecular weight and tendency for self-aggregation has prevented analysis by nuclear magnetic resonance, and the absolute tertiary structure of tubulin remains unknown. The amino acid variations noted among tubulin isotypes are not thought to affect gross protein folding although these differences have been postulated to confer subtle conformational differences, which may alter the dynamics of assembled tubulin (reviewed by Burns, 1991).

An intramolecular interaction between the tubulin carboxyl terminus and a site near the GTP binding site is thought to result in a conformational change which interferes with either GTP binding, hydrolysis, or exchange of GDP for GTP (Padilla et al., 1993; Burns and Surridge, 1990). Biochemical differences within the isotype defining region alone or in combination with posttranslational modifications could result in different molecular conformations which would directly alter tubulin dynamics (Burns and Surridge, 1990; reviewed by Burns, 1991).

Several nomenclature systems for tubulins have emerged, often making it difficult to compare experimental data between systems. Characterization of the complete nucleotide sequences for all β -tubulin isotypes in human, chicken and mouse, has permitted researchers to reconcile these tubulin isoforms

between classification systems. Much of the heterogeneity between tubulin isotypes is localized within the last 50 amino acids. In particular, the last 15 residues of the extreme carboxyl-terminal region of β - (Sullivan and Cleveland, 1986) and to a lesser degree, α -tubulins (Villasante et al., 1986), contain the greatest variation, and are termed "isotype defining" .

These C-terminal amino acid sequence differences have been used to identify six major classes of β -tubulin: I, II, III, IVa and b, V, VI, each with a characteristic pattern of cellular expression (Lopata and Cleveland, 1987). Class VI β -tubulin, found only in red blood cells and platelets, shows the greatest amount of variation, sharing only approximately 75% amino acid sequence homology. The other β -tubulin isotypes vary by only 2 to 8%.

POSTTRANSLATIONAL MODIFICATIONS OF TUBULIN

Isoelectric focusing (IEF) techniques have revealed the presence of more tubulin charge-variants than the known number of tubulin genes. For example, while there are 4 α - and 5 β -tubulin genes expressed in mammalian brain, there are at least 7 α - and 14 β -tubulin isoelectric variants at maturity. Posttranslational modifications of primary gene products generate tubulin "isoforms", and are the source of this tubulin heterogeneity (George et al., 1981; Lee et al., 1990a,b; Eddé et al., 1991). Six posttranslational modifications of tubulin have been characterized to date.

Acetylation

Acetylation of the ϵ -amino group of Lys⁴⁰ of α -tubulin (L'Hernault and Rosenbaum, 1985; Le Dizet and Piperno, 1986) by an acetyl transferase has been identified. This acetyl group can be removed by tubulin deacetylase (LeDizet and Piperno 1987). Acetylated α -tubulin has been found in a variety of differentiated cell types including neurons, muscle and epithelial cells, where it is associated with increased Mt stability (Gundersen and Bulinski, 1986).

Tyrosination/Detyrosination

A reversible modification observed in α -tubulin isotypes which possess a C-terminal tyrosine, is detyrosination and tyrosination. The carboxyl-terminal residue Tyr⁴⁵¹ (Barra et al., 1973) may be removed by carboxypeptidase B (Raybin and Flavin, 1977), and re-attached by tubulin-tyrosine ligase (Ersfeld et al., 1993). Tyrosinated tubulin is present in the soluble pool, while detyrosination occurs primarily on polymerized tubulin. Detyrosination (but not tyrosination) is also associated with increased Mt stability in differentiating cells, and is implicated in cellular morphogenesis (Bulinski and Gundersen, 1991).

Polyglutamylatation

The successive addition of one to six glutamyl residues to the γ -carboxyl group of α -tubulin Glu⁴⁴⁵ (Eddé et al., 1990), β -I Glu⁴⁴¹ and β -VIa tubulin Glu⁴³⁴ (Mary et al., 1994), β -III tubulin

Glu⁴³⁸ (Alexander et al., 1991), and β -II tubulin Glu⁴³⁵ (Rüdiger et al., 1992) can result in the formation of a highly acidic, branched polypeptide tail. Polyglutamylation is the greatest source of tubulin heterogeneity in adult brain, as up to 50% of total α -tubulin, and more than 85% of β -III tubulin is glutamylated (Eddé et al., 1990, Eddé et al., 1991; Alexander et al., 1991). High levels of glutamylated α -tubulin are present in young mouse neurons, while the extent of glutamylation as well as the abundance of glutamylated β -tubulin increases during neuronal development (Audebert et al., 1994). Low levels of glutamylated β -tubulin have been detected in non-neuronal tissues, such as spleen, testis and lung (Wolff et al., 1992).

Polyglutamylation of α -tubulin is not associated with Mt stability, although polyglutamylated β -tubulin exhibits similar dynamics to acetylated tubulin, suggesting it may be correlated with Mt stability (Audebert et al., 1994). Separate enzymes are likely involved in the polyglutamylation of α - and β -tubulin (Wolff et al., 1992).

Deglutamylation of Nontyrosinatable α -Tubulin

Another tubulin modification involves the removal of a tyrosine and glutamic acid residue from the carboxyl terminus of α -tubulin. Tubulin which has undergone removal of Glu⁴⁵⁰ (Paturle-Lafanechère et al., 1991) is referred to as nontyrosinatable or delta 2-tubulin. This modification is found primarily in neurons, where it constitutes 35-50% of brain tubulin and is

thought to be a marker for very stable Mts (Paturle et al., 1989; Paturle-Lafanechère et al., 1991).

Phosphorylation

The kinase pp60^{c-src} has been implicated in the phosphorylation of a tyrosine residue in both α - and β -tubulins present in nerve growth cone membranes and the dynamics of tyrosine-phosphorylated tubulin has been shown to be altered (Matten et al., 1990). Tyrosine phosphorylation of tubulin can be modulated by Ca²⁺-independent neuronal cell adhesion molecules suggesting that this posttranslational modification may provide a mechanism for regulating Mt dynamics at the growth cone (Matten et al., 1990; Atashi et al., 1992). Class III β -tubulin undergoes phosphorylation at Tyr⁴³⁷ (Alexander et al., 1991). Phosphorylation of β -VI Ser⁴⁴¹ (Rüdiger and Weber, 1993) and β -III Ser⁴⁴⁴ (Gard and Kirschner, 1985; Ludueña et al., 1988) has been identified. although the enzyme(s) involved have not been identified. A casein-kinase II-like enzyme which copurifies with brain microtubule proteins is thought to be a likely candidate for β -III tubulin Ser⁴⁴⁴ phosphorylation (Serrano et al., 1987). The temporal regulation of Ser⁴⁴⁴ phosphorylation of neuronal class III (Alexander et al., 1991) has implicated this posttranslational modification in neurite outgrowth.

Glycylation

The carboxyl terminus of axonemal α - and β -tubulins in the ciliate *Paramecium* is modified by the addition of 3 to 34 glycy

units covalently added to Glu⁴³⁷ of β - and Glu⁴⁴⁵ of α -tubulin (Redeker et al., 1994). In bull sperm, approximately 60% of the β -tubulin subunits are modified by the addition of up to 13 glycyI units while α -tubulin is not glycyIated at all (Rüdiger et al., 1995). Nothing is known of the enzyme(s) involved. It remains to be seen whether this modification occurs in other systems.

With the exception of Lys⁴⁰ α -tubulin acetylation, all tubulin modifications take place within the C-terminal region.

ROLE OF THE TUBULIN CARBOXYL-TERMINUS

The greatest variation between β -tubulin isotypes is found in the last 20 amino acids of the carboxyl-terminus (Sullivan and Cleveland, 1986). This region is rich in glutamates and is therefore highly negatively charged (Burns and Surridge, 1990; reviewed by Burns, 1991). Additionally, almost all of the known tubulin posttranslational modifications take place within this region, and contribute to this negative charge.

The C-terminus projects outward from the Mt lattice and is thought to interact with microtubule-associated proteins (MAPs) and Ca²⁺ (Little and Seehaus, 1988; reviewed by Ludueña et al., 1992). Both removal (Serrano et al., 1984) and neutralization of the C-terminus by reaction with carbodiimide promotes Mt polymerization and lowers MAP affinity, suggesting the negative charge present in this region may regulate Mt assembly (Mejillano and Himes, 1991; reviewed by Ludueña et al., 1992).

The C-terminus is thought to contribute to Mt morphology and tubulin dynamics (Padilla et al., 1993; reviewed by Joshi and Cleveland, 1990). For example, β 2 tubulin is necessary for assembly of axonemes in *Drosophila*. When a truncated β 2 protein missing the last 15 amino acids is expressed, it assembles into Mts, but is not incorporated into axonemal structures. This truncated β 2 tubulin protein is also less stable than the endogenous β 2 tubulin (Fackenthal et al., 1993).

The C-terminus is thought to be free to interact with the tubulin GTP binding site, possibly altering GTP binding or hydrolysis and directly influencing dynamic instability (Burns and Surridge, 1990; Padilla et al., 1993). Biochemical differences within the isotype-defining region alone, or in combination with posttranslational modifications may therefore affect molecular conformations and consequently tubulin dynamics (Burns and Surridge, 1990). When the tubulin isotypes present in brain are purified and assembled, each tubulin isotype exhibits unique Mt dynamics *in vitro* (Banerjee et al., 1988; Banerjee et al., 1990; reviewed by Ludueña, 1992) and *in vivo* (Panda et al., 1995).

Additionally, the C-terminus is thought to play a role in the folding of the tubulin molecule following translation, and may stabilize tubulin intermediates by interacting directly with the GTP binding site (Fontalba et al., 1995). Isotype-specific differences in the rate of tubulin folding and dimer formation have been identified (Zabala and Cowan, 1992).

REGULATION OF CELLULAR TUBULIN LEVELS

Transcriptional activation is the first stage at which cellular tubulin levels are regulated. In transfection experiments involving higher eukaryotes, the highest levels of tubulin expression have been achieved when endogenous tubulin promoter sequences are used to drive tubulin expression. In *Drosophila*, when the 5' promoter-containing sequences of the major testis-specific $\beta 2$ tubulin gene was used to drive expression of the developmentally regulated $\beta 3$ gene, $\beta 3$ tubulin levels constituted more than 20% of endogenous tubulin. This suggests that these non-coding regions contain elements which may be important in regulating tubulin transcription (Hoyle and Raff, 1990; reviewed by Murphy, 1991).

In cells such as fibroblasts, tubulin expression is thought to be constitutive (Cleveland and Havercroft, 1983). However, the cell-specific pattern of tubulin gene expression (Lopata and Cleveland, 1987) suggests that molecular genetic mechanisms are in place which coordinate gene expression with differentiation. For example, the rat neuronal $T\alpha-1$ α -tubulin promoter is sufficient to confer neuron-specificity to a $T\alpha 1:lacZ(n)$ transgene during development and regeneration (Miller et al., 1994). Analysis of the specific promoter elements implicated in this neuron-specific activation is underway.

Post-transcriptional mechanisms are thought to regulate the level of tubulin mRNA in developing rat brain and selective stabilization of specific isotypes of tubulin mRNA has been observed. The steady state mRNA levels of rat β -II tubulin (the

major neuronal tubulin isotype) at postnatal day 5 were 168-fold greater than adult levels, while the transcription rate was only 3.6 fold greater at postnatal day 5 than in adult brain (Moskowitz and Oblinger, 1995). Thus, processing of tubulin mRNA during neurogenesis appears to provide another level of tubulin regulation (Bhattacharya et al., 1991).

During translation, appropriate cellular levels of unassembled β -tubulin are autoregulated via the strictly conserved first 4 translated codons MREI (met-arg-glu-ile) sequence of the β -tubulin amino terminus (Yen et al., 1988). Excess soluble β -tubulin, along with an unidentified effector molecule (Theodorakis and Cleveland, 1992) are thought to bind to the first 4 amino acids of the nascent polypeptide (which must be at least 41 codons long), resulting in destabilization of the tubulin mRNA, and termination of translation (Cleveland et al., 1981; Cleveland and Havercroft, 1983).

α -tubulin is thought to undergo autoregulation independent of β -tubulin (Bachurski et al., 1994), although the mechanism by which this is accomplished is unknown. When a β -tubulin isotype normally expressed in Chinese hamster ovary (CHO) cells was epitope-tagged with the hemagglutinin antigen and expressed in CHO cells, the expression of the endogenous β -tubulin isotype decreased, but α -tubulin expression increased (Gonzalez-Garay and Cabral, 1995). This result suggests that there is co-regulation of α - and β -tubulin levels within these cells.

A posttranslational level of tubulin regulation appears to exist which can selectively degrade tubulin isotypes. When

chicken Class IV β -tubulin was over-expressed in CHO cells, transcription of the chicken Class IV mRNA increased fourfold relative to endogenous tubulin isotypes while the Class IV isotype did not accumulate to greater than 10% total tubulin. A selective decrease in endogenous Class IV β -tubulin transcription was also noted (Sisodia et al., 1990).

FUNCTION OF TUBULIN POLYMORPHISM

The functional significance of tubulin heterogeneity remains unclear. One view holds that isotype usage is indiscriminate with all tubulin isoforms expressed within a cell uniformly incorporated into all Mts (Raff, 1984). This implies one isotype may be substituted for another without loss of function. A variety of transfection studies support this view (reviewed by Ludueña et al., 1993; reviewed by Raff, 1995). For example, a chimeric chicken-yeast β -tubulin was prepared by replacing the chicken C-terminal sequence with an extremely divergent yeast C-terminal sequence. When this mutant tubulin was expressed in 3T3 fibroblast cells it was incorporated into all Mt arrays, with no apparent loss of function (Bond et al., 1986). Similarly, transfection of the erythrocyte-specific β VI tubulin in HeLa cells (Lewis et al. 1987) yielded similar results suggesting the functional equivalence of tubulin isotypes.

The "multi-tubulin" hypothesis proposed by Fulton and Simpson (1976) suggests that specific tubulin isotypes expressed within a cell are necessary to form Mts with a particular

function. The conservation of both sequence and pattern of expression noted within vertebrate β -tubulins argues that selection has played a role in the maintenance of isotype specificity (Sullivan and Cleveland, 1986; Sullivan, 1988). The tissue specificity, temporal expression and posttranslational modification of some tubulin isotypes supports the idea that cytoplasmic distribution and Mt properties may be influenced by isotypic differences.

In *Drosophila*, when the developmentally regulated minor β 3 isotype is expressed in place of the major isoform β 2 in testes, some of the resultant Mts exhibit loss of function. When β 3 was expressed either alone or at levels exceeding 20% of the total tubulin pool, axoneme assembly was disrupted, resulting in male sterility (Hoyle and Raff, 1990) suggesting that the two β -tubulin isotypes are not functionally equivalent and that isotype differences play a role in the formation of different Mt arrays.

Similarly, both the 67C and 84B α -tubulins in *Drosophila* are required for normal spindle function in the oocyte and early embryo, and thus are not functionally equivalent (Matthews et al., 1993). In *Caenorhabditis*, six touch-receptor neurons have Mts with 15 protofilaments. When the tissue-specific β -tubulin gene *mec-7* is mutated, the number of protofilaments drops to 11, and the animals are touch-insensitive (Savage et al., 1989).

Whether tubulin isotypes are functionally equivalent, or are in fact necessary to form functionally distinct Mts remains unclear. Non-specialized cells which express few tubulin

isotypes do not appear to exhibit isotype discrimination. However, in more specialized cells, such as neurons, the developmental expression of MAPs and posttranslational modification of specific tubulin isotypes suggests isotypic distinctions may exist in these cells (Cambray-Deakin, 1990; Joshi and Cleveland, 1990).

MICROTUBULES AND CELLULAR MORPHOGENESIS

Actively proliferating cells contain a radial array of dynamic Mts which extend from the MTOC and grow out towards the periphery of the cell through the preferential addition of tubulin to the (+) end. These Mts exhibit dynamic instability, with some Mts growing rapidly while others are experiencing sudden catastrophic disassembly (Mitchison and Kirschner, 1984). During cellular morphogenesis, however, postmitotic cells, such as neurons, establish a less dynamic or more stable subpopulation of Mts which may not be centrosome-based (Black and Greene, 1982; Lim et al., 1989; Falconer et al., 1989a, 1992). These more stable Mts exhibit a slow rate of tubulin loss. The resultant asymmetrical Mt cytoskeleton generates a unique cellular polarity (Mitchison and Kirschner, 1984; Kirschner and Mitchison, 1986).

Posttranslational modifications have been implicated in the selective stabilization of Mts during cellular morphogenesis. A model for morphogenesis suggests that an extracellular signal may generate Mt stabilization sites at specific location(s) within the cell. This initial stabilization permits these Mts

to remain polymerized long enough to undergo some form of polymer-associated posttranslational modification (Kirschner and Mitchison, 1986; Bulinski and Gundersen, 1991). Acetylation, detyrosination, and polyglutamylation of tubulin have been shown to accumulate concomitantly with the increased Mt stability associated with neuronal differentiation (Cambray-Deakin and Burgoyne, 1987; Black et al., 1989; Audebert et al., 1994).

Such biochemical modifications may permit cell proteins, such as structural and motor MAPs, for example, to interact with a selected population of Mts. In this way, specific Mts could be targeted for further stabilization through enhanced MAP binding affinities, or perhaps serve as a marker or "track" for organelle localization or intracellular transport. Such a mechanism may explain the observation that only 25% of the Mts present in lobster axons are utilized for vesicle transport (Miller et al., 1986). Different posttranslational modifications found within the same cell may act as signals for a variety of distinct cellular activities (Bulinski and Gundersen, 1991).

At present, the mechanism(s) by which posttranslational modifications of tubulin result in increased Mt stability is unknown, it is only known that the two events are correlated.

MICROTUBULE-ASSOCIATED PROTEINS

Tubulin can be purified from an homogenized vertebrate brain extract through a polymerization-depolymerization cycling process. A group of proteins, known as microtubule-associated

proteins (MAPs) co-purify with Mts isolated from brain and associate with Mt polymer *in vivo* and *in vitro* (Weingarten et al., 1975).

MAPs are involved in both the assembly and the stabilisation of Mts (reviewed by Matus, 1991; Littauer et al., 1986). They form fine filamentous cross-bridges which appear to cross-link neuronal Mts (Lee and Brandt, 1992) and suppress the dynamic instability of Mts (Pryer et al., 1993; Dhamodharan and Wadsworth, 1995). Two major classes of structural MAPs have been characterized: high molecular weight MAPs (270-350 kD) which include MAP1A, MAP1B and MAP2 and MAPs with a molecular weight between 55-82 kD, including tau proteins, chartins and STOP (stable tubule only protein) proteins (reviewed by Matus, 1991).

Expression of tau, Map2 and Map2c by transfection or microinjection in cells which do not normally express these MAPs results in an increase in stability and bundling of Mts (reviewed by Chapin and Bulinski, 1992; Schoenfeld and Obar, 1994). Differences in MAP/tubulin binding affinities have been observed. For example, the binding affinity of MAP2 to chicken brain Mts is twofold higher than to erythrocyte Mts, suggesting that MAPs may interact preferentially with specific tubulin isotypes (reviewed by Murphy, 1991).

Differences in the subcellular distribution of MAPs have been reported (reviewed by Hirokawa, 1991; Matus, 1988). MAP2 is found within dendrites, while tau proteins are thought to be sequestered within axons (reviewed by Matus, 1988). Similarly, differences in the localization of posttranslationally modified

MAPs have been noted. For example, MAP1B is known to be present in both axons and dendrites, but is phosphorylated within developing axons only (Diaz-Nido et al., 1991). Inter-Mt distances vary between axons and dendrites and it is not known if spacing variation is the result of differences between MAPs (reviewed by Hirokawa, 1990). Posttranslational modifications of MAPs may provide an additional mechanism to regulate the association of MAPs with tubulin (reviewed by Matus, 1988).

Two structurally-related MAPs exhibit complementary patterns of expression during neuronal development. Levels of MAP1B peak during neuronal development and decline following maturation, while MAP1A appears much later during development and persists in mature neurons (Schoenfeld et al., 1989). Expression of MAP2 and MAP2c expression is similarly regulated during neurogenesis, with MAP2c expressed at high levels only during neuronal development, and MAP2 present only in mature neurons (reviewed by Matus, 1988).

The high level of expression and developmental regulation of MAPs suggests that these cytoskeletal proteins play an important role in regulating the differential stability exhibited by neuronal Mts (Cambray-Deakin et al., 1990; reviewed by Matus, 1991).

MICROTUBULE ORGANIZATION IN NEURONS

Neurons are highly specialized cells with long processes which extend from the cell body to form contacts with target cells. Neuronal Mts must be tractable to permit neurite

outgrowth, respond to environmental cues and injury, and yet sufficiently stable to maintain established neural circuitry (Lim et al., 1989; Baas and Black, 1990). Temporal and spatial changes in Mt dynamics are associated with the development and maintenance of neuronal processes (reviewed by Mitchison and Kirschner, 1988; Joshi and Baas, 1993).

Mts within neuronal processes are discontinuous with the centrosome, existing in short longitudinal segments along the shaft. During neurite outgrowth, the (+) or growing ends of all Mts are oriented towards the periphery of the cell but a change in polarity occurs with the differentiation of axons and dendrites (Baas et al., 1989). In mature neurons, microinjection of biotin-labelled tubulin demonstrates the preferential addition of label to the fast-growing (+) ends of Mts (Okabe and Hirokawa, 1988) which in the axon, are uniformly oriented distal to the neuronal soma (Heidemann et al., 1981). In dendrites, short Mts exhibit mixed polarity, with 50% of (+) ends proximal to the cell body and 50% distal (Baas et al., 1988).

Along the neurite shaft there is a progressive stabilization of Mts (Lim et al., 1989); however, even within the shaft of the axon the distal (+) ends of Mt segments are labile while the proximal regions become progressively more stable (Baas and Black, 1990) and serve as nucleating sites for Mts within the axon during growth (Baas and Ahmad, 1992).

Mts in the growth cone are highly dynamic (Tanaka and Kirschner, 1991) and function in both growth cone motility and

axonal growth (Bamburg et al., 1986; Tanaka and Kirschner, 1995; Tanaka et al., 1995). These Mts are highly sensitive to Mt-depolymerizing drugs (Bamburg et al., 1986).

The molecular mechanisms regulating the differential stability of Mts in neurons is not known. Both association with MAPs and posttranslational modifications of tubulin, however, have been correlated with increased Mt stability.

CLASS III β -TUBULIN

Of the β -tubulin isotypes expressed in mammalian brain, Class III β -tubulin is expressed exclusively in neurons (Frankfurter et al., 1986; Moody et al., 1989) and exhibits the greatest amino acid sequence divergence, differing from the others by 10% (reviewed by Sullivan, 1988). Expression of β -III tubulin in chick and rat embryo neuroblasts is reported to coincide with the final mitotic event (Moody et al., 1989; Lee et al., 1990a; Memberg et al., 1995) and increased levels of β -III tubulin have been noted in regenerating dorsal root ganglion cells (Moskowitz and Oblinger, 1995) suggesting a functional role in neurite outgrowth.

While low levels of Class III β -tubulin are found in the Sertoli cells of testes (Lewis and Cowan, 1988), it does not appear to undergo Ser⁴⁴⁴ phosphorylation in these cells (Lee et al., 1990b; Frankfurter et al., 1986). Ser⁴⁴⁴ phosphorylation of β -III tubulin (and phosphorylation of MAP1B, and tau-like proteins) occurs during neurite extension, suggesting that regulatory factors may be involved in tubulin polymerization,

and implicating the phosphorylation of cytoskeletal proteins in neurite outgrowth (Diaz-Nido et al., 1991). Dephosphorylated $\alpha\beta$ III tubulin exhibits a reduced ability to polymerize in the presence of MAP2 *in vitro* (Khan and Ludueña, 1994).

When PC-12 cells are induced to extend neurites, the level of β -III tubulin expression is unchanged, but a redistribution of β -III tubulin from the cell body to the growing neurites occurs. β -III tubulin is assembled at a lower efficiency than the other β -tubulins, and exhibits a patchy distribution along the Mts (Asai and Remolona, 1989; Joshi and Cleveland, 1989). Additionally, Falconer et al. (1992) demonstrated the preferential sorting of Class II β -tubulin into colchicine-stable Mts, with an apparent exclusion of Class III β -tubulin shortly after neural induction of P19 cells.

The *in vitro* polymerization properties of isotypically-purified β -III tubulin have been shown to be intrinsically different from β -II and β -IV tubulin, which are also expressed in neurons. Class III β -tubulin demonstrates slower kinetics when induced to assemble in the presence of either MAPs or 4 M glycerol and 6 mM CaCl_2 (reviewed by Ludueña, 1993). Increased Mt assembly has been noted in brain tubulin depleted of β -III (Banerjee et al., 1990) and the colchicine-binding kinetics of β -III tubulin has been shown to differ from the other β -tubulin isotypes expressed in neurons (Banerjee and Ludueña, 1992).

The distinctive properties of this tubulin isotype suggest that β -III tubulin may have a specific functional role in the establishment and maintenance of neuronal architecture.

NEURONAL DIFFERENTIATION MODEL

Cultured cells can serve as convenient *in vitro* model systems to observe events of neurogenesis which may be obscured within the mammalian embryo. A number of cell lines are widely used to study neuronal development and function. Neuroblastoma cell lines, deprived of serum, express many neuronal proteins, and exhibit striking morphological differentiation as the normally rounded cells extend long neurites. Pheochromocytoma (PC-12) cells grown in the presence of nerve growth factor (NGF) exhibit morphology and physiology typical of sympathetic neurons (reviewed by Banker and Goselin, 1991).

These cell lines are maintained in a neuronally-committed state, and therefore do not permit observation of the earliest events of neurogenesis. Additionally, neuroblastoma and PC-12 cells are not terminally differentiated and will retract neurite extensions in the absence of morphogens.

Primary cultures of hippocampal neurons isolated from embryonic rat brain and grown in culture provide a further model to study neurogenesis. These cells, however, do not proliferate in culture, and new cells must be extracted for each new experiment (reviewed by Banker and Goselin, 1991).

Of the tissue culture model systems available, this laboratory has chosen to use the P19 embryonal carcinoma cell line.

P19 EMBRYONAL CARCINOMA CELLS

P19 embryonal carcinoma (EC) cells were isolated from a

teratocarcinoma which was generated when a 7 day mouse embryo was grafted to the testis of a mouse. The clonal cell line derived from this tissue and maintained in culture at an exponential growth rate remains undifferentiated. At high cell densities, these euploid EC cells spontaneously differentiate into a heterogenous population of tissue cells, including muscle, epithelial, neuronal, glial and cartilage (McBurney and Rogers, 1982; reviewed by McBurney, 1993).

In the presence of 0.5%-1% dimethylsulphoxide (DMSO) P19 cells differentiate into beating cardiac muscle, and with 2% DMSO, into smooth muscle (McBurney et al., 1982). P19 cells treated with 10^{-6} M retinoic acid (RA) differentiate to neurons, glia and fibroblast-like cells. The terminally-differentiated, postmitotic neurons derived from this culture system, have been shown to express neuronal antigens and display typical morphology with long neuritic processes extending from small cell bodies (Jones-Villeneuve et al., 1982; McBurney et al., 1988; reviewed by McBurney, 1993).

This multipotent cell system permits investigation of the earliest events in the development of the neuronal cytoskeleton and provides an abundant and reproducible source of material for analysis throughout differentiation. The potential for differentiation into muscle, provides a useful control when studying neuron-specific gene expression during development (Falconer et al., 1992). Undifferentiated P19 cells are easily transfected with foreign DNA (reviewed by McBurney, 1993).

This pluripotent cell line was used as a differentiating

model system (day 0 to day 12) to characterize the expression, posttranslational modification, and colchicine-stability of Class III β -tubulin during neurogenesis.

RATIONALE FOR EXPERIMENTS

Falconer et al. (1992) used immunofluorescence microscopy and immunoblotting, with antibodies to Class I, II, III, and IV β -tubulin isotypes to characterize the sorting of these β -tubulin isotypes in neurally-induced and in muscle-induced P19 cells. Total Mt arrays were compared with colchicine-stable arrays (those arrays which were resistant to depolymerization following incubation with 1 μ g/mL colchicine for 45 min). Several changes in β -tubulin isotype sorting were noted. Shortly after neural-induction, there was a preferential sorting of Class II β -tubulin into colchicine-stable Mts, with an apparent exclusion of Class III β -tubulin. In muscle cells, while β -II tubulin is present, it was β -IV which was preferentially incorporated into stable Mt arrays.

I am interested in the mechanisms by which Mts are selectively stabilized during neuronal differentiation. The restricted expression, distinctive biochemical properties, and unique Ser⁴⁴⁴ phosphorylation of β -III tubulin suggests that this tubulin isotype may be functionally important in the dramatic cytoskeletal remodelling that occurs during neuronal morphogenesis.

HYPOTHESIS:

The selective utilization of Class III β -tubulin isoforms directly or indirectly plays a role in the development of stable Mts in developing P19 neurons.

TEST OF HYPOTHESIS:**1. Characterization of β -III tubulin in P19 neurons**

To elucidate a specific role for β -III tubulin in neurogenesis, the sorting of β -III tubulin and its posttranslationally modified isoforms into dynamic or stable populations of Mts in P19 neurons was characterized. Using a monoclonal antibody to Class III β -tubulin (TuJ1) (Frankfurter et al., 1986; Lee et al., 1990a) and a combination of immunofluorescence staining, immunoblotting of isoelectric focusing gels, enzyme-linked immunosorbent assay and northern blotting, the expression, posttranslational modification and colchicine-stability of this tubulin isotype in undifferentiated (day 0) and differentiating (days 3, 6, and 12) cells was examined.

2. Effects of taxol on Class III β -tubulin

In undifferentiated P19 cells the Mt system is highly dynamic and few Mts are resistant to colchicine treatment (Falconer et al., 1989a, 1989b). A low level of Class III β -tubulin is expressed in some cells, but it is not detected in assembled Mts. At day 2, Class II β -tubulin expression is up-regulated and polymerized β -III tubulin is first detected in a

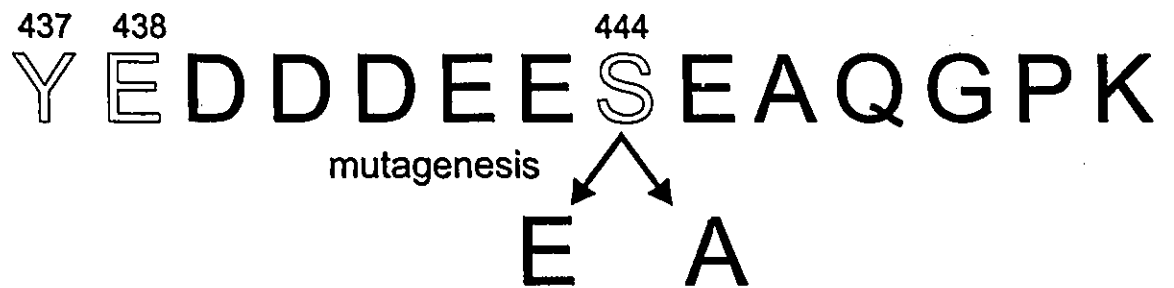
subset of cells which have not yet extended neurites. β -III tubulin is predominantly found in the soluble fraction of these early neurons and there is a preferential sorting of β -II tubulin into colchicine-stable Mts and an apparent exclusion of β -III tubulin. By day 3, however, neurite outgrowth is evident and β -III tubulin is detected in colchicine-resistant Mts in developing neurons (Falconer et al., 1992).

The alkaloid, taxol, alters Mt dynamics *in vivo* and *in vitro* by promoting the assembly of tubulin into Mt polymer that is stable to depolymerization by cold or CaCl_2 (Schiff et al., 1979; Nogales et al., 1995). In this study the effects of taxol on the organization and incorporation of β -III tubulin into the Mt systems in both undifferentiated and neuronally-induced P19 cells was examined by immunofluorescence microscopy and isoelectric focusing followed by immunoblotting.

3. Expression of Class III β -tubulin in Undifferentiated P19 cells

To test the significance of β -III tubulin Ser⁴⁴⁴ phosphorylation, wild-type β -III tubulin and two β -III tubulin mutants (Ser⁴⁴⁴ replaced by Ala⁴⁴⁴; and Ser⁴⁴⁴ replaced by Glu⁴⁴⁴ in another) (Figure 1) were constructed and expressed in undifferentiated P19 cells. Both the assembly competency and colchicine-stability of Mts containing these β -III tubulins was assessed by immunofluorescence microscopy.

Figure 1. Posttranslational modifications of β -III tubulin present in the C-terminus of β -III tubulin and proposed mutagenesis of Ser⁴⁴⁴ to Glu⁴⁴⁴ (E) and Ala⁴⁴⁴ (A).



Phosphorylation of Tyr⁴³⁷
Phosphorylation of Ser⁴⁴⁴
Polyglutamylation (1-6 residues) of Glu⁴³⁸

METHODS AND MATERIALS

CELL CULTURE

Undifferentiated P19 cells were maintained at 37°C and 5% CO₂ in α -modified Eagle's minimal essential medium (α -MEM) (Gibco BRL, Burlington, ON) and 10% heat-inactivated fetal calf serum (FCS) (Oxoid, Nepean, ON) with a passage interval of 48 hr. Cultures seeded at 3 X 10⁵ cells/mL in 100-mm tissue-culture grade dishes (CanLab) experience a 10-fold increase in number in 48 hr (Robertson, 1987).

When passing cells, growth medium was removed and the cells rinsed using calcium and magnesium-free phosphate-buffered saline (PBS). To facilitate removal of cells from the culture dish surface, the cells were incubated for 10 min in 1.5 mL 0.25% trypsin (w/v) (Sigma) and 1 mM ethylenediamine tetraacetate (EDTA) (Sigma) in PBS at 37°C and then vigorously pipetted to provide a single cell suspension. PBS (8.5 mL) was added and cells were counted using a BrightLine Hemocytometer.

For immunofluorescence experiments, 18-mm glass coverslips were seeded with 8 X 10⁴ cells and cultured for 24 hr in growth medium to provide a semi-confluent layer of cells. 100-mm culture dishes were seeded with 5 X 10⁵ cells for extractions.

After 24 hr, a monolayer of day 0 cells was available. To induce neural differentiation 10⁻⁶ M retinoic acid (RA) was added to the growth medium for 24 hr. This was replaced by defined medium (MacPherson and McBurney, 1995) and changed every 48 hr.

DRUG TREATMENTS

Colchicine

A stock solution of 1 mg/mL colchicine (Sigma) in sterile distilled water was stored at -80°C . For all experiments, this stock was diluted 1:1000 in culture medium to provide a final concentration of 1 $\mu\text{g}/\text{mL}$ colchicine and added to cell cultures under 5% CO_2 at 37°C , for time periods indicated.

Taxol

Taxol (obtained from the National Cancer Institute, Bethesda, MD) was dissolved in DMSO to provide a 1×10^{-2} M stock and further diluted in α -MEM at concentrations from 1×10^{-9} M to 1×10^{-6} M taxol so that DMSO did not exceed 0.3% v/v. 0.3% v/v DMSO was added to control cultures. Cell cultures were treated with the appropriate concentration of taxol diluted in growth medium, for 24 hr at 37°C .

CELL FIXATION

Several fixation methods were employed, but for all methods, the coverslips displaying a semi-confluent layer of cells were first briefly rinsed with calcium- and magnesium-free PBS at 37°C .

Simultaneous extraction/fixation:

Cells were incubated 5 min in 0.5% Triton X-100 in Mt-stabilizing buffer (0.1 M N-morpholinoethanesulfonic acid, pH 6.75, 1 mM MgSO_4 , 2 mM EGTA, 0.1 mM EDTA, 4 M glycerol) at 37°C to extract soluble proteins (Figure 2) and then fixed with paraformaldehyde (3.7%)/glutaraldehyde (0.25%) and 1% Triton X-100 in PEM (40 mM PIPES, 2.5 mM EGTA, 0.5 mM MgCl_2) [all from Sigma] for 10 min at RT. Coverslips were then rinsed 3 X 5 min in PBS.

Figure 2. Tubulin extraction illustrating polymeric and soluble tubulin fractions. The antibody YOL 1/34 has been used to label soluble and polymeric tubulin in undifferentiated P19 cells which have been fixed without extraction of soluble tubulin (A). Following extraction with 0.5% Triton X-100, only Mt polymer remains (B).

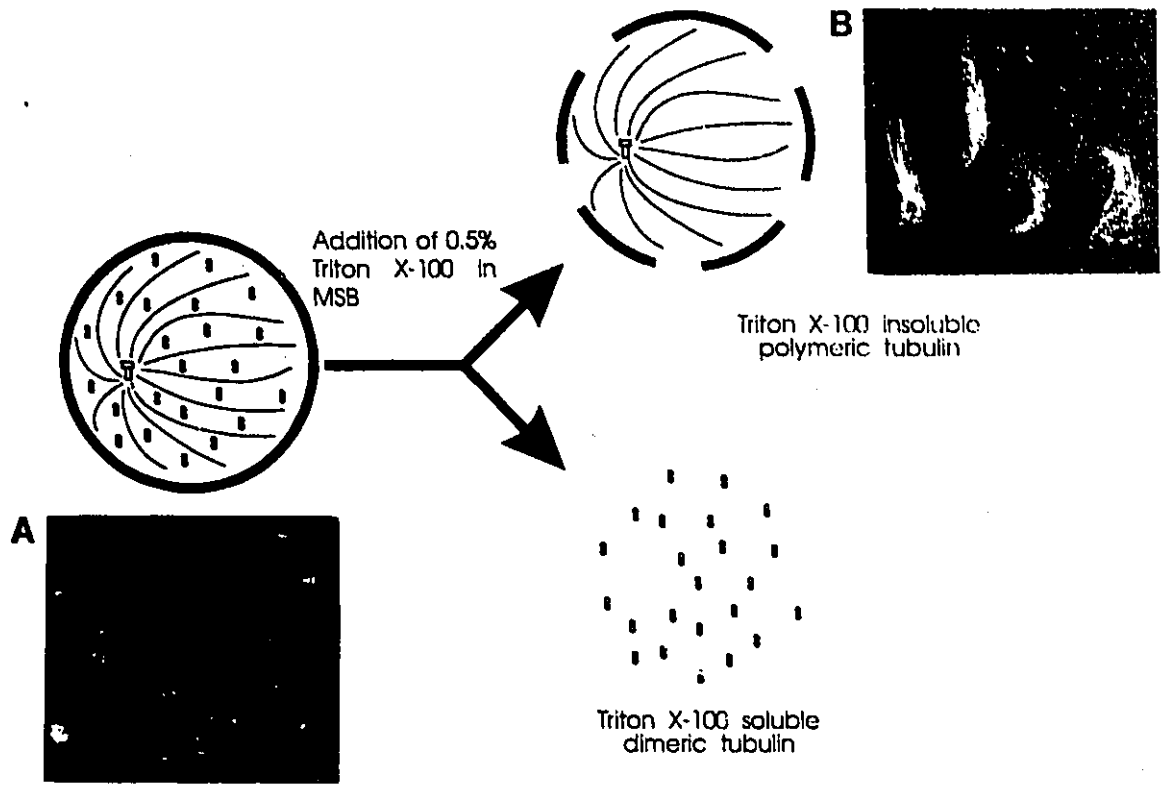


Figure 1. Preparation of extracts containing soluble and polymeric tubulin.

Methanol:

Cells growing on coverslips were immersed in absolute methanol for 20 min at -20°C , followed by 3 X 5 min rinses in PBS.

Lana's Fixative:

Cells growing on coverslips were immersed in Lana's fixative (4% paraformaldehyde, 14% saturated picric acid (v/v) in 0.25 M NaH_2PO_4 , pH 6.9) for 45 min at RT, followed by 3 X 5 min rinses in PBS. The cells were then extracted for 5 min, with 0.5% Triton-X 100 in PBS at RT and rinsed 2 X 5 min with PBS.

PRIMARY ANTIBODIES

Anti- α -tubulin (VOL 1/34) (Sigma): rat monoclonal (IgG) which recognizes α -tubulin was diluted 1:200 for immunofluorescence microscopy.

Anti- β -tubulin (DM1B) (Amersham, Oakville, ON): a mouse monoclonal (IgG) which recognizes all β -tubulin isotypes was diluted 1:1000 for immunoblotting, and quantitative ELISA determinations.

Anti- β -III tubulin (TuJ1) (gift from Dr. A. Frankfurter): a mouse monoclonal (IgG) which recognizes Class III β -tubulin, was diluted 1:300 for immunofluorescence microscopy, 1:500 for immunoprecipitation, and 1:1000 for immunoblotting and ELISA.

SECONDARY ANTIBODIES

SECTIONS A AND C: (Figures 7,8,18,20 and 21):

1. Donkey anti-rat (IgG) conjugated to fluorescein

isothiocyanate (FITC), cross-adsorbed to mouse (Jackson, Bio/Can Scientific, Mississauga, ON), diluted 1:150.

2. Donkey anti-mouse (IgG) conjugated to indocarbocyanine (CY3), cross-adsorbed to rat (Jackson), diluted 1:500.

SECTION B: (Figures 11,14,15, and 16):

1. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rat (IgG) cross-adsorbed to mouse (Chemicon), diluted 1:100

2. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (IgG) cross-adsorbed to rat (Zymed, Dimension Labs, Mississauga, ON), diluted 1:80.

IMMUNOFLUORESCENCE LABELLING

All cell experiments involved simultaneous double-immunolabelling with two primary antibodies diluted in PBS and incubated with fixed cells for 45 min at RT. Coverslips were then rinsed 3 X 5 min in PBS, followed by simultaneous incubation with two secondary antibodies diluted in PBS for 45 min at RT. Cells were stained for 1 min in Hoechst dye no. 33258 (Calbiochem-Behring Corp., La Jolla, Calif.) diluted 1:10000 (w/v) in PBS, to stain DNA. This was followed by 3 X 5 min rinses in PBS. Coverslips were mounted in medium containing 2.5% (w/v) 1,4-diazabicyclo(2.2.2.)octane (DABCO, Aldrich Chem. Corp., Milwaukee, WI) dissolved in 9 parts glycerol to 1 part Tris buffer, pH 8.6.

For all experiments, labelled cells were examined using a Zeiss Axiophot microscope equipped with epifluorescence and phase contrast optics, and photographed using Ilford XP2 400 film.

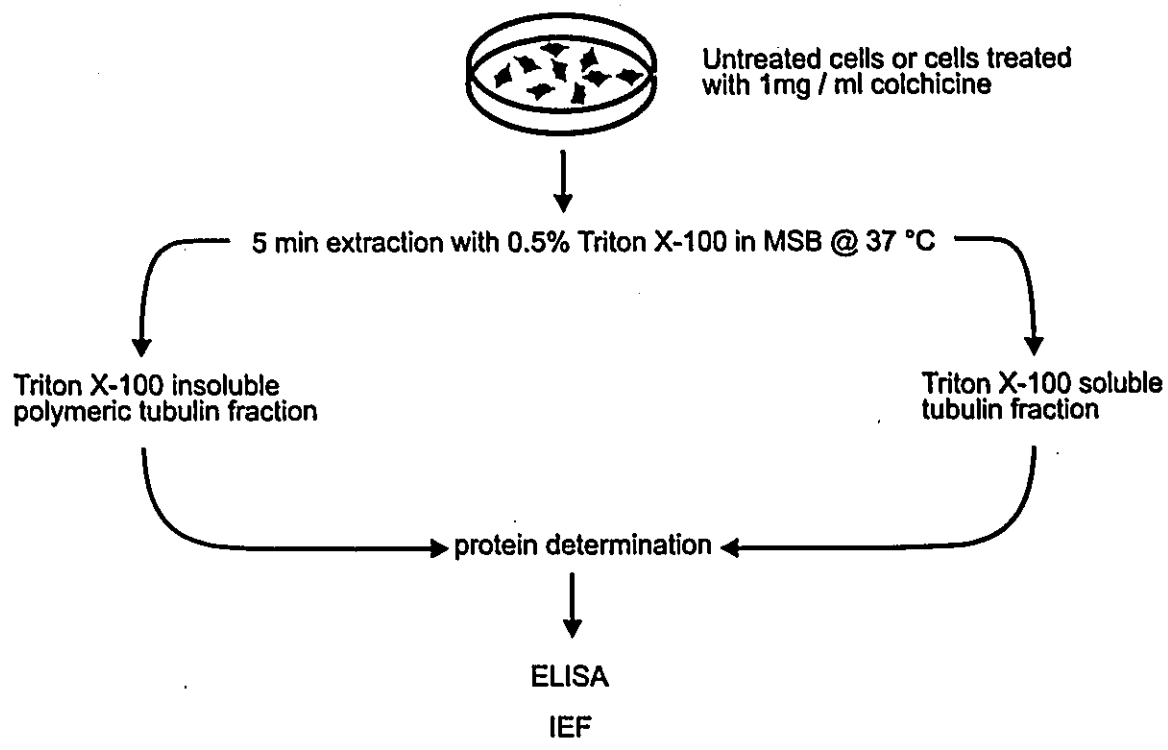
NORTHERN BLOTTING

Total RNA was extracted (at intervals indicated) by the LiCl Urea extraction procedure of Auffray and Rougeon (1980). 10 μ g of total RNA was denatured and run on each lane of a 1% formaldehyde gel for 6 hr. The samples were transferred by capillary action to a nylon filter, Hybond N (Amersham) and the samples crosslinked to the membrane with a GS Genelinker UV crosslinker (Biorad) at 125 mJ. Blots were hybridized in 5X SSPE [NaCl/Na₂HPO₄/EDTA], 50% formamide, 0.5% SDS, 5X Denhardt's (Denhardt, 1966) and 250 μ g/mL salmon sperm DNA overnight at 42°C. The probes were labelled with α P³² dCTP (Dupont/NEN) with the multiprime labelling kit (Dupont). Blots were washed at high stringency (0.1X SSC [1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate], and 0.1% SDS) for 30 min. Membranes were exposed to Kodak XAR film for 1-3 d. The β -III tubulin probe (kindly provided by Dr. A. Frankfurter) is an Apal/MscI fragment cloned into Bluescript KS+ (cut with Apal/SmaI) containing the last 3 residues of the mouse Class III β -tubulin gene and extending about 150 bases into the 3'UTR. To verify equal RNA loading the full-length sequence of the β -actin cDNA (kindly provided by Dr. M. McBurney, University of Ottawa, Ottawa) was also used.

PREPARATION OF CELL EXTRACTS

This procedure is summarized in Figure 3. At each day of differentiation, the semi-confluent monolayer of cells was very briefly rinsed with microtubule stabilizing buffer (0.1 M N-

Figure 3. Summary of polymer and soluble tubulin sample preparation for ELISA and IEF.



morpholinoethanesulfonic acid, pH 6.75, 1 mM MgSO₄, 2 mM EGTA, 0.1 mM EDTA, 4 M glycerol) at 37°C. Soluble proteins were extracted for 5 min in 500 µL of microtubule stabilizing buffer containing 0.5% Triton X-100 at 37°C (Figure 2). The soluble extract was removed and centrifuged at 13,000 rpm for 2 min to pellet any residual cytoskeletal material. 500 µL of solubilizing solution (0.5% SDS, 25 mM TRIS, pH 6.8) was added to the polymeric fraction remaining in the dish along with the cytoskeletal pellet isolated from the soluble extract (Joshi and Cleveland, 1989). The following protease inhibitors were added to all solutions: 2 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin A, 2 µM aprotinin, and 100 µM leupeptin [all from Sigma]. Lysates (in microfuge tubes) were heated in a boiling water bath for 10 min, followed by centrifugation at 13,000 rpm for 2 min. Total protein in each supernatant was determined by bicinchoninic acid assay (Smith et al., 1985). Samples were stored at -80°C.

To obtain colchicine-treated cell extracts, 1 µg/mL colchicine (Sigma) was added to a companion dish of cells for time periods indicated prior to extraction. Cells were then treated exactly as described above.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The modified method of Voller et al. (1979) was used with a Ceres UV900HDi scanning autoreader (Bio-Tek Instruments Inc., Winooski VT). The primary antibodies TuJ1 and DM1B were diluted 1:1000, a biotinylated goat anti-mouse antibody (Amersham) was

diluted 1:1000 followed by a streptavidin/HRP complex (1:750) [Amersham]. Colour development was with o-phenylenediamine (Sigma). Samples were prepared as described in Figure 3. Phosphocellulose-purified tubulin (Weingarten et al., 1975) was isolated from bovine brain for use as a standard for tubulin determinations. 1-2 μg of protein from each fraction was applied to Immulon I flat bottom microtitre plates (Dynatech Lab, Chantilly, VA) assayed by ELISA, and then normalized to reflect the proportion of total protein present in the extract sample. (The protein value from the polymeric and soluble fraction from each dish of cells was added together to determine total cell protein and the ELISA value was multiplied by the percentage of total protein present in each fraction). β -III tubulin values were expressed as a percentage of the maximum day 12 absorbance value. Each sample was analyzed in triplicate.

ISOELECTRIC FOCUSING

Samples were prepared as described above (Figure 3). Following protein determination, cell extracts were precipitated by adjusting extracts to 80% acetone (-20°C), mixed with a vortex mixer, and left at -20°C for 15 min. Precipitated proteins were pelleted by centrifugation at 13,000 rpm for 30 min at 4°C (Lee et al., 1990a). Acetone was removed, and protein was dissolved in sample buffer (9.16 M urea, 5% β -mercaptoethanol, 2.5% Ampholine pH 4-7 [Pharmacia, Baie D'Urfé, PQ], 2% NP-40 and a few grains of bromophenol blue (purchased from Canlab) to provide a protein concentration of 10 $\mu\text{g}/\mu\text{L}$.

Immobiline DryStrip gels pH 4-7 (Pharmacia) were rehydrated overnight in 50 mL freshly prepared rehydrating solution (8.5 M urea, 1% NP-40, 0.075 g dithiothreitol, and 1 M acetic acid and aligned on an LKB Multifor 1 apparatus (Pharmacia). Either equal amounts of protein (200 μ g) or equal amounts of total tubulin (20 μ g), measured by ELISA, were loaded at the cathode, and electrofocused 3 hr at 300 V followed by 16 hr at 2300 V.

A series of proteins with characterized isoelectric points (Pharmacia) were used to calibrate the pH gradient profile across the IEF gel.

IMMUNOBLOTTING

Proteins were transferred to nitrocellulose by capillary action (Elkon, 1984), blocked for 2 hr with 2% milk powder in PBS and immunolabelled using TuJ1, followed by biotinylated horse anti-mouse, IgG vector (1:1000) [Sigma], and finally streptavidin/HRP (1:5000) [Amersham]. Binding was detected by enhanced chemiluminescence (ECL) [Amersham] using HyperFilm-ECL (Amersham).

Following ECL detection of β -III tubulin, the nitrocellulose membrane was washed 2 X 10 min in PBS and stripped with 2% SDS, 100 mM mercaptoethanol in 62.5 mM Tris-HCL (pH 6.8) for 30 min at 70°C, followed by 2 X 10 min in PBS (according to the manufacturers' protocol). Immunoblotting was then performed as described above, only this time, the membrane was probed with the 1° antibody DM1B diluted 1:1000 in PBS.

³²PO₄ LABELLING OF P19 CELLS

Cells were grown in 100-mm dishes to the appropriate day of differentiation, as previously described. The α -MEM was then replaced with phosphate-reduced DMEM (Dulbecco's minimal essential medium) (Gibco BRL, Burlington, ON). 500 μ Ci/mL ³²PO₄ (HCl and Carrier Free) (NEN, Dupont) was added, and cells incubated approximately 20 hr in 5% CO₂ at 37°C. This medium was then removed and cells rinsed briefly in PBS at 37°C.

IMMUNOPRECIPITATION

A modification of the method of Afar et al. (1992) was used. 250 μ L boiling 1% SDS was added to each 100-mm dish, and cells scraped into eppendorf tubes. 750 μ L cold immunoprecipitation buffer (10 mM Tris, 150 mM NaCl, 2 mM NaF, 2 mM Na pyrophosphate, 500 mM NH₄PO₄, 2 μ L/mL aprotinin, 5 μ g/mL leupeptin, 1% Triton X-100), 10 μ L PMSF and 50 μ L vanadate was added to each tube, and extract mixed by inversion. 300 μ L of this mixture was removed from each sample to provide a sample control (no 1° antibody added).

TuJ1 (1:500) was added to each sample, mixed by inversion, and incubated on ice for 1 hr. Rabbit anti-mouse (1:750) [Cappel] was added to all samples (including control) and incubated on ice for 1 hr. 50 μ L of Protein A Sepharose (Pharmacia) was added and mixed on a rotating mixer for 1 hr at 4°C. Samples were centrifuged at 13,000 for 1 min, and supernatant discarded. The pellet was rinsed 5 times, by resuspending in cold immunoprecipitation buffer and centrifuging

at 13,000 for 1 min. After the final rinse, 40 μ L IEF sample buffer was added, samples were vortexed, placed in a boiling water bath for 2 min, and centrifuged 1 min at 13,000. 30 μ L of the sample was added to each IEF gel as described previously.

Following electrofocusing, proteins were transferred to nitrocellulose by capillary action (Elkon, 1984) and the membrane exposed to Kodak XAR film for 48 hr at -80°C . Immunoblotting with TuJ1 was then performed as described previously except that color development was with 4-chloro-1-naphthol (BioRad).

PLASMID DNA

The cDNA coding for mouse β -III tubulin (m β 6) was kindly provided by Dr. N. Cowan, New York University Medical Centre, New York, NY. To verify that the sequence was complete, sequencing of the 5' (approx. 300 bp) and 3' (approx. 500 bp) ends was performed using the chain termination method T7 Sequenase Kit (Promega, Madison, WI). The complete β -III tubulin coding sequence, including approximately 75 bases of 5' UTR and 380 bases of 3' UTR was then used to make the following constructs.

1. *Pgk-1*- β -III tubulin in pUC19

The HindIII/XbaI fragment containing the complete cDNA coding and 3' untranslated sequence for mouse β -III tubulin, was blunt-ended and cloned into the SmaI site of the plasmid pKJ1 R (kindly provided by Dr. M. McBurney, University of Ottawa,

Ottawa). The plasmid pKJ1 R contains the mouse *Pgk-1* (3-phosphoglycerate kinase) promoter cloned into the plasmid pUC19 (Figure 4).

2. *Pgk-1- β -III TUBULIN Ala⁴⁴* in pUC19

β -III cDNA in M13mp15, carrying the desired mutation was introduced into the vector *Pgk-1- β -III* by subcloning a 1.1 kb *Nco1/Bsp120I* fragment from the M13mp15 mutated β -III into the *Pgk-1- β -III* tubulin vector from which the wild type *Nco1/Bsp120I* fragment had been removed (Figure 4).

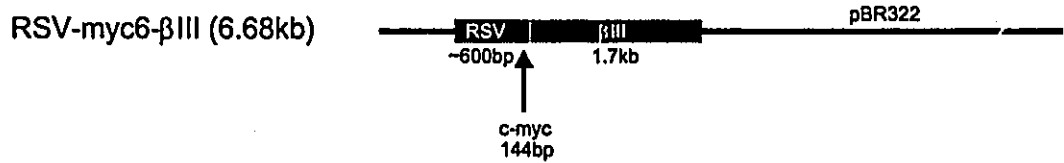
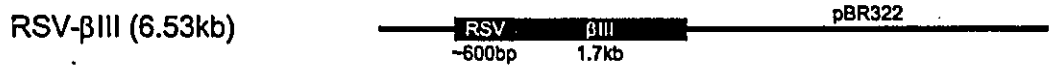
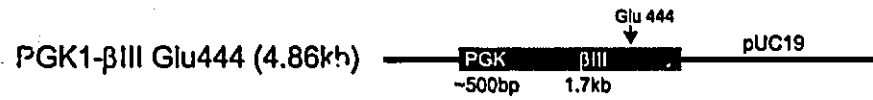
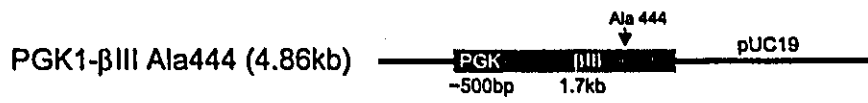
3. *Pgk-1- β -III TUBULIN Glu⁴⁴* in pUC19

β -III cDNA in M13mp15, carrying the desired mutation was introduced into the vector *Pgk-1- β -III* by subcloning a 1.1 kb *Nco1/Bsp120I* fragment from the mutated β -III into the *Pgk-1- β -III* tubulin vector from which the wild type *Nco1/Bsp120I* fragment was removed (Figure 4).

4. *RSV- β -III tubulin* in pBR322

The complete cDNA coding and 3' untranslated sequence for mouse β -III tubulin was first cut with *Xba1*, blunt-ended and then digested with *HindIII*. The 1.7 kb fragment was cloned into the *HindIII/Sma1* site of the plasmid pRSV (kindly provided by Dr. M. McBurney, University of Ottawa, Ottawa). The plasmid pRSV contains the RSV (Rous sarcoma virus) promoter cloned into the plasmid pBR322 (Figure 4).

Figure 4. Summary of plasmids.



5. RSV-6myc- β -III tubulin in pBR322

The cDNA sequence for mouse β -III tubulin was first cut with *Apa*I (which removed the 5' UTR along with the first 12 bp of the coding region), blunt ended and subsequently ligated with a 144 base pair human 6-myc oligonucleotide (kindly provided by Dr. M. McBurney, University of Ottawa, Ottawa) to provide β -III tubulin which is missing the first 4 amino acids and has an immunological 'tag' at the 5' end. This 6-myc- β -III construct was then digested with *Xba*I/*Xho*I and cloned into the blunt-ended *Hind*III/*Bgl*II site of the plasmid pRSV (kindly provided by Dr. M. McBurney, University of Ottawa, Ottawa). The plasmid pRSV contains the RSV (Rous sarcoma virus) promoter cloned into the plasmid pBR322 (Figure 4).

In addition, the expression vector *Pgk-1-lacZ* in pUC19 was kindly provided by Dr. M. McBurney, University of Ottawa. *lacZ* is a bacterially derived gene which codes for the enzyme β -galactosidase. This enzyme will metabolize the chromophore (X-gal) to produce a blue colour, and was used as a reporter gene to assess transfection efficiency.

PREPARATION OF PLASMID DNA

Bacterial transformations, large-scale and mini-preparations of plasmid DNA, and DNA restriction mapping methods were all performed according to Sambrooke et al., 1989. Briefly, for large scale preparations, each plasmid was used to transform competent DH5 α F' *E. coli* (kindly provided by Dr. M.W. McBurney, University of Ottawa, Ottawa) using heat-shock, and

cultures were then plated on Terrific (T)-broth agar (Difco Laboratories, Detroit, MI) plates containing 50 $\mu\text{g}/\text{mL}$ ampicillin (Sigma), and grown overnight at 37°C. Colonies were picked and used to inoculate 5 mL T-broth containing 50 $\mu\text{g}/\text{mL}$ ampicillin, and grown overnight with vigorous agitation at 37°C. 2 mL of this liquid culture was used to inoculate 250 mL of T-broth containing 50 $\mu\text{g}/\text{mL}$ ampicillin, and the culture incubated overnight, with vigorous agitation at 37°C. Large-scale preparation of DNA was by the alkaline-lysis method (Birnboim and Doly, 1979) [all reagents used were from Sigma, except SDS and sodium perchlorate which were obtained from BDH, Toronto, ON].

Concentration of plasmid DNA was determined by spectrophotometric analysis of UV absorbance. Preparations were determined to be free of RNA and protein contamination, with A_{260}/A_{280} ratios between 1.721 and 1.791. Plasmid DNA was stored at -80°C.

SITE-DIRECTED MUTAGENESIS

In order to generate the single-stranded (ss) DNA required for the mutagenesis protocol the 1.7 kb HindIII/XbaI fragment containing the complete cDNA coding and 3' untranslated sequence for mouse β -III tubulin, was subcloned into the HindIII/XbaI site of the M13mp15 vector (kindly provided by Dr. D. Johnston, University of Ottawa, Ottawa). This vector can exist in two forms: a replicative (double-stranded) plasmid and non-replicative (single-stranded) phage form. The ssDNA isolated

was sequenced. Mutations of β -III tubulin were introduced using a site-directed mutagenesis protocol. To prepare β -III tubulin mutants, GCT (coding for Ser⁴⁴⁴) was replaced by either CGC (coding for Ala⁴⁴⁴) or CTG (coding for Glu⁴⁴⁴) (Figure 1) using reagents and instructions supplied in the "Sculptor in vitro mutagenesis system" (Amersham) and the following oligonucleotides (purchased from the Biotechnology Institute, University of Ottawa, Ottawa).

1. Ser⁴⁴⁴ to Ala⁴⁴⁴ (S to A)

5' CCC TGG GCT TCC GCT TCC TCG TCA TC 3' CGC = Ala⁴⁴⁴

2. Ser⁴⁴⁴ to Glu⁴⁴⁴ (S to E)

5' GGC CCC TGG GCT TCC TGT TCC TCG TCA TCA 3' CTG = Glu⁴⁴⁴

Briefly, for each mutation, an oligonucleotide (approximately 26 nucleotides) with the necessary base(s) mismatch to the complementary β -III tubulin cDNA sequence was phosphorylated.

This phosphorylated oligonucleotide was then annealed to ss β -III in M13mp15 and used to prime DNA synthesis *in vitro*. T4 DNA ligase and T7 DNA polymerase were then added, along with thiol-nucleotides (dNTP) to permit extension and ligation of the mutant strand. T5 exonuclease was then added to the extension reaction to remove any single-stranded, non-mutant DNA which did not undergo extension in the previous step, leaving only the heteroduplex molecule.

The non-mutant cDNA strand was removed by using a restriction enzyme (NciI) to nick the non-mutant strand of the heteroduplex (the mutant strand was protected from this enzyme

by the presence of the thiol-nucleotides), and exonuclease III to digest non-mutant DNA from the site of mutation. Finally, dNTPs, DNA polymerase I and T4 DNA ligase was added to repolymerize the gapped DNA and provide double-stranded closed circular DNA, providing a homoduplex of mutant molecules. Mutants were identified by sequencing (automated dideoxy-sequencing generously provided by Dr. M. Tenniswood, W. Alton Jones Centre, Lake Placid, NY)

PROTOCOLS FOR M13 PHAGE

All procedures involving M13 phage were performed as described by Messing (1983) and are briefly described below.

Phage Preparations

Each plaque was grown in 1.5 mL aliquots of an exponential culture of DH5 α F' in 2 X T broth at 37°C and incubated for 6 hr at 37°C using a shaker. The tube was then centrifuged at 9,000 rpm for 10 min at 4°C and the supernatant transferred to an eppendorf tube, taking care not to transfer any of the pellet.

Isolation of Phage DNA

200 μ L of polyethylene glycol/NaCl was added to 1.0 mL of phage supernatant at RT for 30 min, followed by centrifugation for 10 min at 13,000 rpm. The entire supernatant was carefully removed and discarded. The pellet was resuspended in 100 μ L of tris/EDTA (TE), and 50 μ L of phenol, and the sample vortexed for 10 sec and incubated for 10 min at RT. The sample was then

vortexed an additional 10 sec and centrifuged for 3 min at 13,000 rpm. The supernatant was first extracted with 50 μ L of phenol/chloroform, vortexed 10 sec and spun 3 min at 13,000 rpm and subsequently with 50 μ L chloroform in a similar fashion. The supernatant was then precipitated overnight with 50 μ L of ammonium acetate and 250 μ L ethanol at -20°C . To recover the precipitate the sample was centrifuged for 10 min at 13,000 rpm and washed twice with 80% ethanol, dried, resuspended in 25 μ L of TE, and stored frozen, at -20°C .

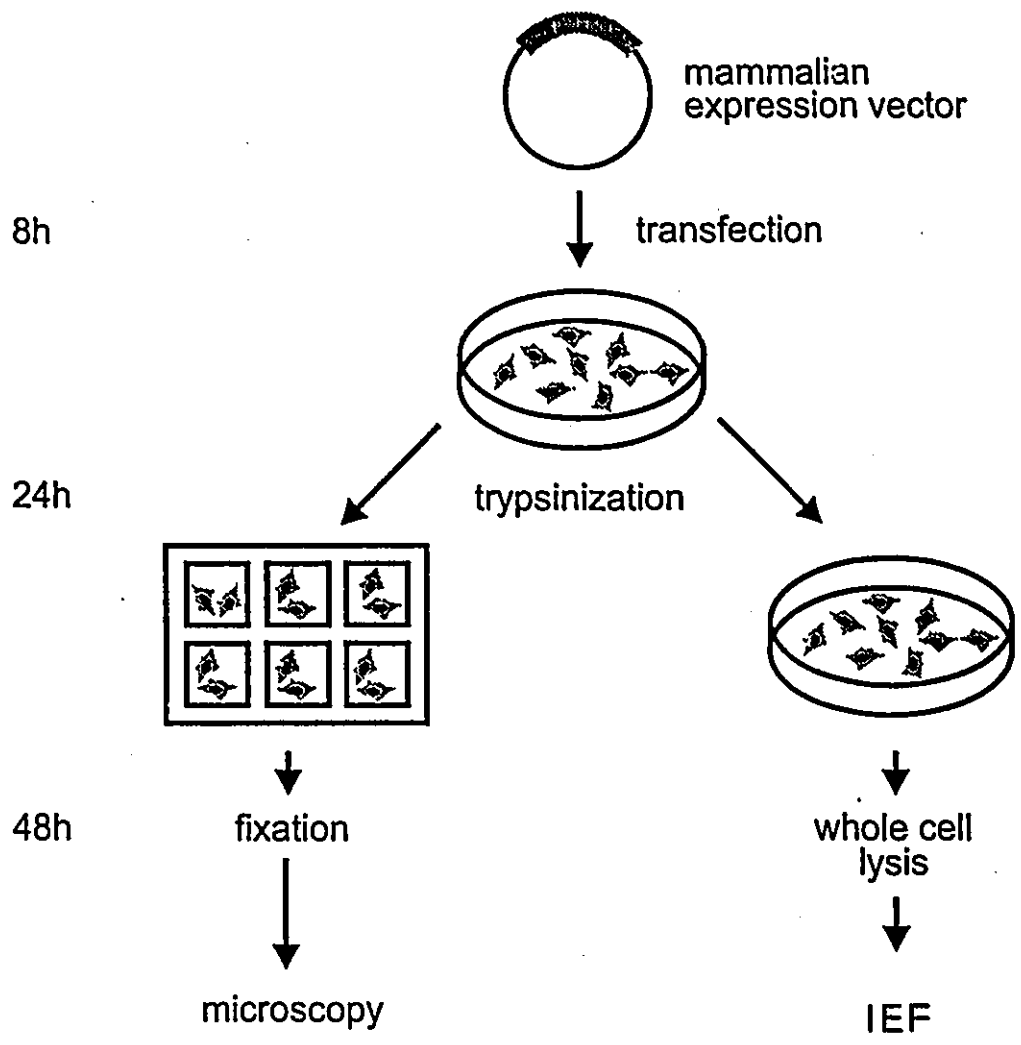
CaPO₄/DNA PRECIPITATION TRANSFECTION

Plasmid DNA was introduced into P19 cells by transfection using the calcium-phosphate precipitation method described by Chen and Okayama (1987) except that cells were incubated with the DNA/calcium phosphate solution for 8 hr at 37°C under 5% CO₂ (Figure 5). 13 μ g of the plasmid carrying the gene of interest along with 500 μ L 0.25 M CaCl₂ and 500 μ L 2X BES buffer was added to each 60-mm dish of semi-confluent cells in all transfections. To determine transfection efficiency, 2 μ g of the plasmid pDJM2 (kindly provided by Dr. M. McBurney, University of Ottawa, Ottawa) which contains the *Pgk-1* promoter and the bacterial *lac-Z* cDNA cloned into the plasmid pUC19 was co-transfected. Transfected cells were identified by microscopy following X-gal histochemical staining or by immunofluorescence microscopy using an antibody to the protein encoded by the gene of interest. Transfection efficiencies ranged from 10 to 30%.

X-GAL HISTOCHEMICAL STAINING

Cells were fixed in 0.2% glutaraldehyde in PBS for 20 minutes and rinsed 2 X 5 min in PBS. X-gal solution (Dannenberg and Suga, 1981) was added and cells incubated overnight at 37°C. The X-gal reagent is a substrate for β -galactosidase, and its presence results in the deposition of blue crystals in those cells containing *E. coli* β -galactosidase.

Figure 5. Summary of transient transfection protocol.



RESULTS

A. CHARACTERIZATION OF CLASS III β -TUBULIN IN P19 NEURONS

1. Class III β -Tubulin Expression During Differentiation of P19 Neurons

As an initial step in the characterization of Class III β -tubulin expression in P19 neurons, β -III tubulin mRNA expression was examined by preparing total RNA extracts from differentiating P19 cells. Twenty-four hr following the addition of RA, β -III tubulin mRNA was barely detectable, with levels gradually increasing to day 6 (Figure 6). By day 12, a reduction in β -III mRNA to slightly more than day 3 levels was evident. Examination of β -III tubulin mRNA levels between day 6 and day 12 revealed a peak at day 7 (data not shown). Total RNA extracts from three separate time-course experiments yielded similar results.

To examine the expression of the β -III tubulin protein and its distribution in Mts within developing neurons, RA-induced P19 cells were examined by immunofluorescence microscopy. Undifferentiated P19 cells (day 0) exhibited typical centrosome-based Mt arrays labelled by YOL 1/34, and demonstrated no polymerized β -III tubulin (Figure 7a-a''). Forty-eight hr following the addition of RA, β -III tubulin appeared in the mitotic spindle as well as in apparently typical interphase Mt arrays of neuronal precursors (Figure 7b-b''). By day 3 many neurons could be detected growing over a monolayer of non-neuronal cells. These neurons exhibited β -III labelling of Mts

Figure 6. Northern blot analysis of β -III tubulin mRNA expression. Total RNA (10 μ g) prepared from undifferentiated (day 0) and RA-induced (days 1 to 12) P19 cells and probed with a β -III tubulin probe (upper frame). β -III tubulin mRNA increases from day 1 until approximately day 6, at which point levels begin to decrease. Probing with β -actin to confirm that approximately equal amounts of RNA were loaded (lower frame). Localization of ribosomal RNA transcripts (rRNA), 28S (approx. 5000 nucleotides) and 18S (approx. 2000 nucleotides) are shown.

β III-TUBULIN mRNA EXPRESSION IN P19 EC CELLS

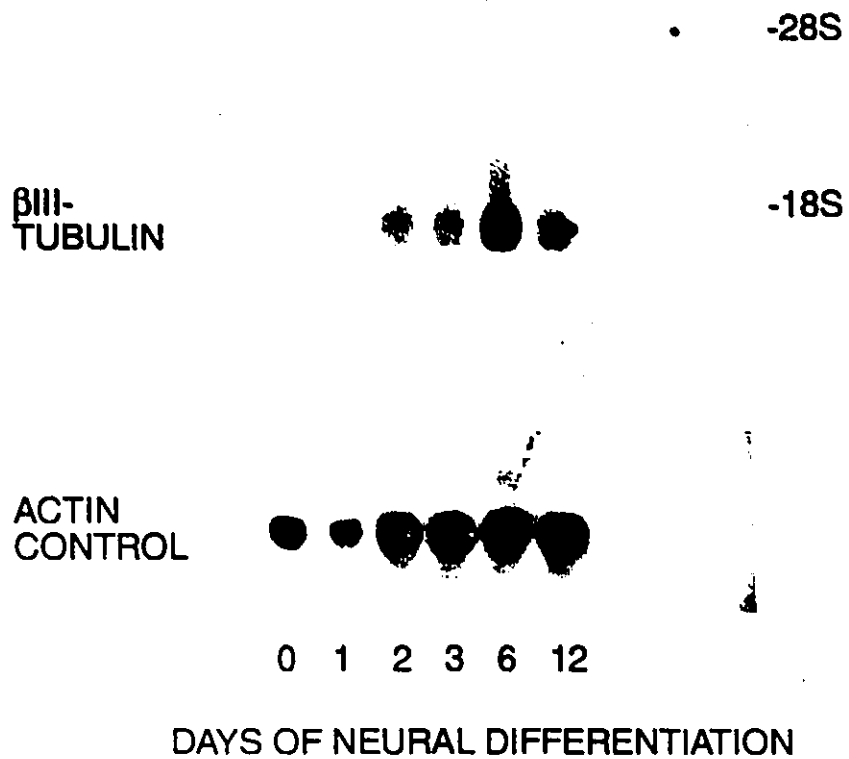
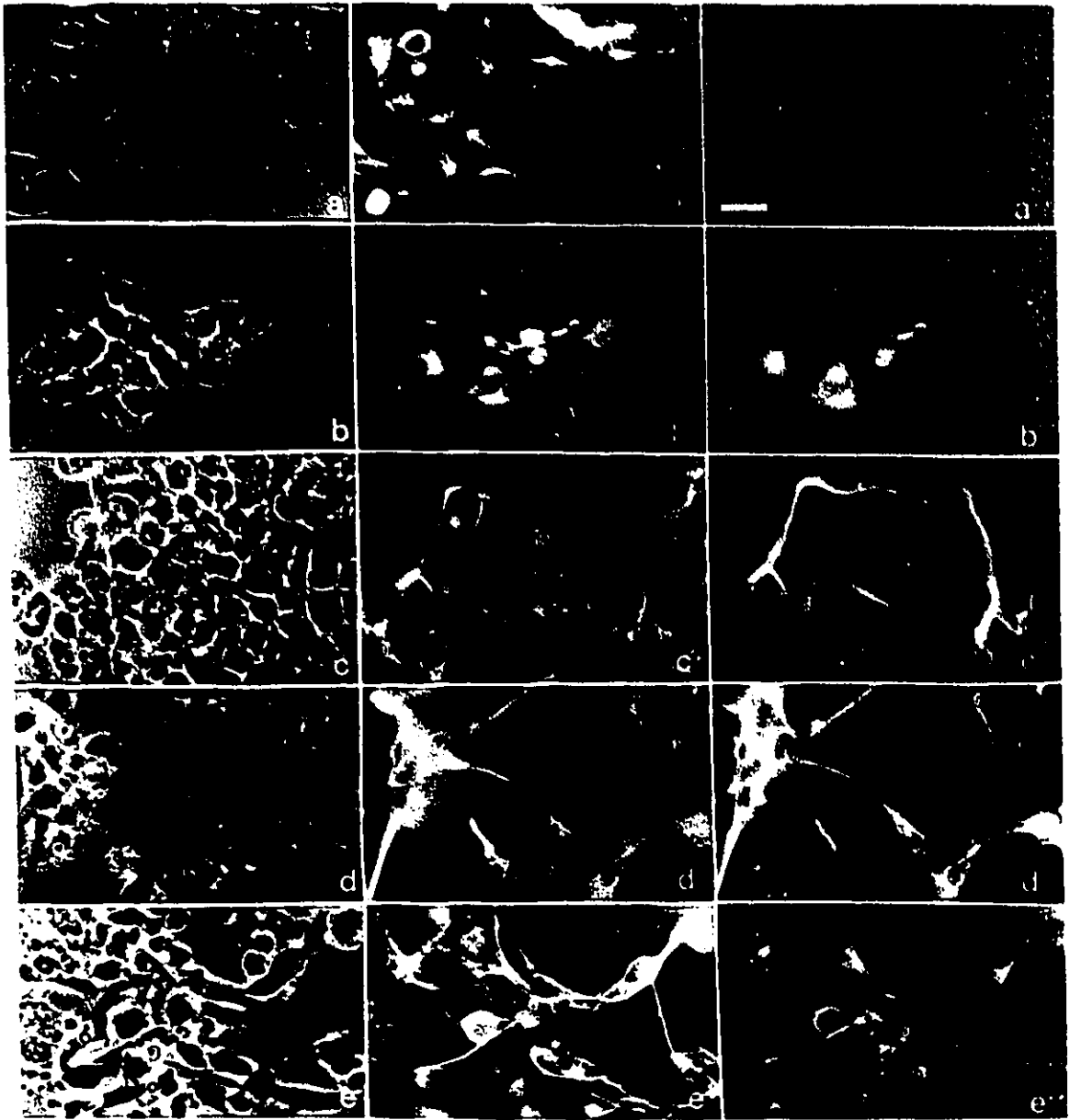


Figure 7. Phase contrast (a-e) and double-immunofluorescence micrographs of Mts in RA-induced P19 cells. Cells were double-labelled using a monoclonal antibody to α -tubulin (a'-e') and a monoclonal antibody to β -III tubulin (a''-e''). No polymerized β -III tubulin is present in undifferentiated P19 cells (a''). By day 2, β -III tubulin was detected in the mitotic spindle (arrow) of neuroblasts and in cells without neurites (b''). The progressive elaboration of neuronal Mts was evident in day 3 (c'), day 6 (d'), and day 12 (e'), with β -III Mts found (c'',d'',e'') within neuronal cell bodies, and along increasingly more arborized neurites. Bar, 10 μ m.



within the cell body, and along newly extended, relatively broad neurites. Most neurons were unipolar although some displayed bipolar processes with apparently uniform labelling of β -III tubulin in all extensions (Figure 7c-c''). In day 6 neurons, β -III tubulin appeared to be incorporated into Mts within the cell bodies and along the now predominantly bipolar, thinner, neuronal processes. Neuronal cell bodies were present in distinct aggregates, with neurites extended outward towards other clusters of neurons (Figure 7d-d''). By day 12, the neuronal culture consisted largely of organized aggregates of neuronal cell bodies with parallel neurites running between them, making microscopic examination of individual neurons more difficult. The more sparsely populated field shown here (Figure 7e-e''), demonstrates that while extensive β -III labelling of Mts was evident throughout the neurons, the intensity of the signal appeared diminished (Figure 7e'') from that of the β -III labelling at day 6 (Figure 7d'').

2. Colchicine-Stability of Microtubules Increases Following Neuronal Differentiation

To investigate the subcellular localization of β -III tubulin in stable Mts undifferentiated (day 0) and differentiating (day 2-12) P19 cells were exposed to colchicine treatments of 45 and 90 min. (Mts containing β -III tubulin will be referred to hereafter as β -III Mts, although it is clear that these Mts also contain other available β -tubulin isotypes). No β -III Mts remained after 45 min colchicine treatment of day 0 to

day 2 cells (data not shown). At day 3, only neurons which had undergone neurite outgrowth exhibited stable β -III Mts. After 45 min of colchicine, these Mts were found extending from the cell body along the length of the neurites (Figure 8a'-a''). Following 90 min of colchicine, β -III labelling of Mts within the cell body was reduced to a narrow bundle which appeared continuous with stable Mts along the neurite (Figure 8b'-b''). Mts in non-neuronal cells in the culture were completely disassembled after 45 or 90 min colchicine treatment. In day 6 neurons treated with colchicine for 45 or 90 min, β -III tubulin could be found along the neurites of many neurons; however, an apparent decrease in intensity of β -III labelling compared to general Mt labelling with YOL 1/34 was noted (Figures 8c'-c'' and 8d'-d''). In addition to Mt polymer, apparent aggregates of β -III tubulin which remained associated with the cytoskeleton following extraction were noted. By day 12, more β -III tubulin remained associated with the cytoskeletal fraction as apparent aggregates of β -III tubulin as well as in β -III Mts following both colchicine-treatments (Figure 8e'' and 8f'').

The results of the northern analysis and the immunofluorescence staining indicated that the levels of both the β -III mRNA and protein decreased at the later stages of neuronal differentiation. To examine this quantitatively enzyme-linked immunosorbent assay (ELISA) was used to determine the absolute amount of total tubulin (α - and β -) and the relative amount of β -III tubulin present in the polymer and soluble tubulin fractions of RA-induced P19 cells (Figure 9A and

Figure 8. Phase contrast (a-f) and double-immunofluorescence micrographs of Mts in RA-induced P19 cells following colchicine-treatment (45 and 90 min). Cells were double-labelled using a monoclonal antibody to α -tubulin (a'-f') and a monoclonal antibody to β -III tubulin (a''-f''). General Mt labelling (a' and b') and β -III Mts (a'', b'') in neurons exhibit colchicine-resistance at day 3. By day 6, numerous Mts (c', d'), including many labelled by anti- β -III (c'', d''), remain following colchicine treatment. Day 12 colchicine-stable Mts are evident (e', f'), many of which are labelled by anti- β -III (e'', f''). Bar, 10 μ m.

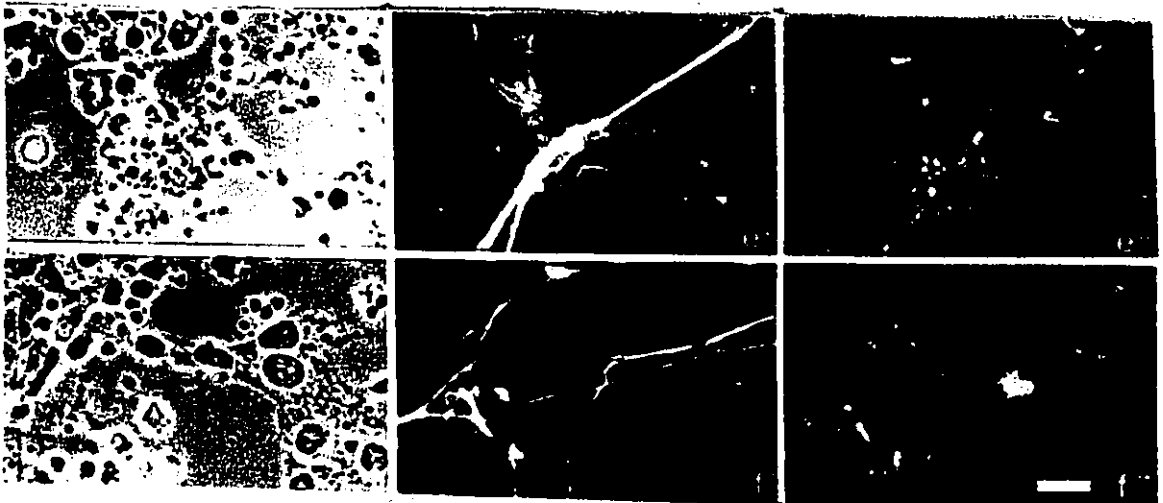
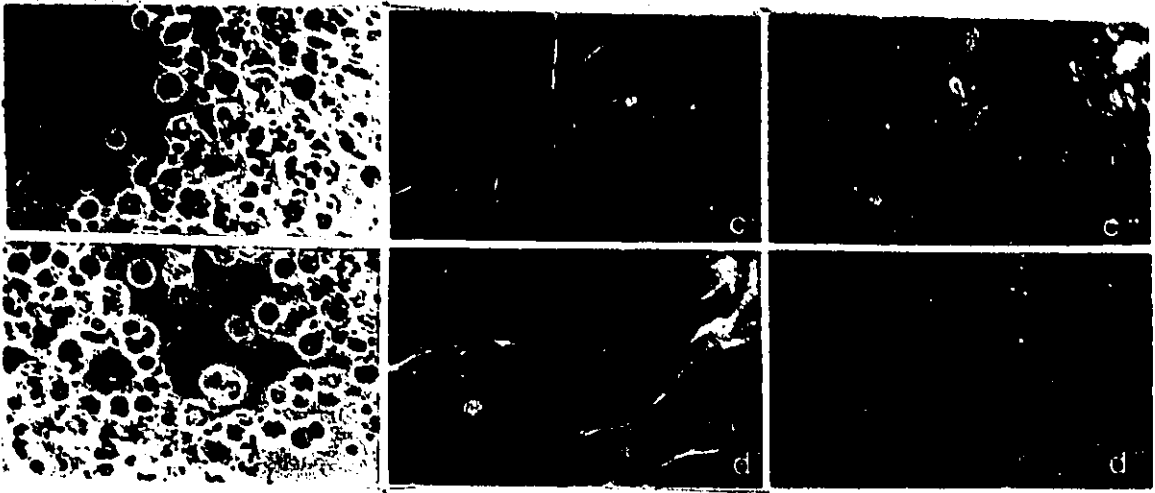
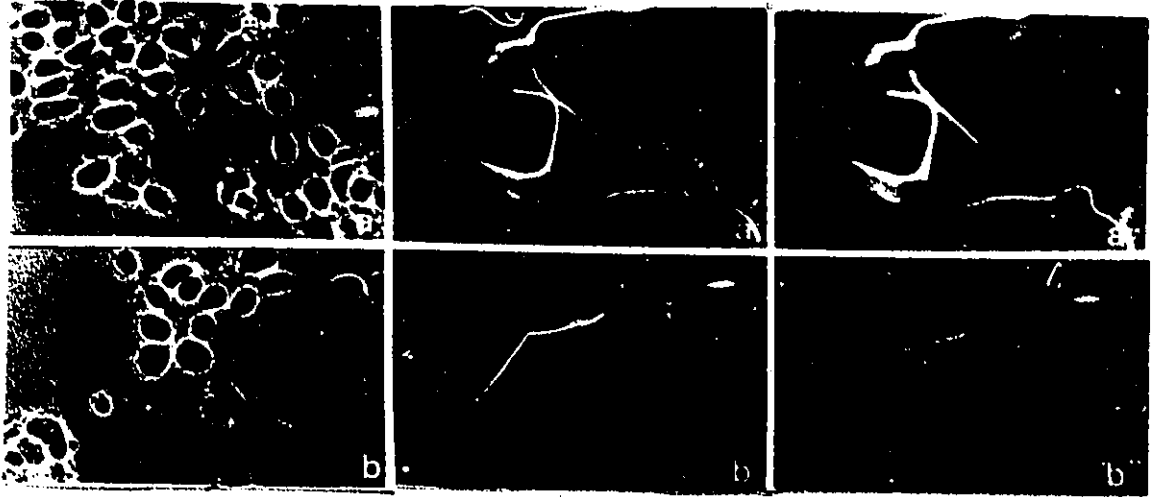
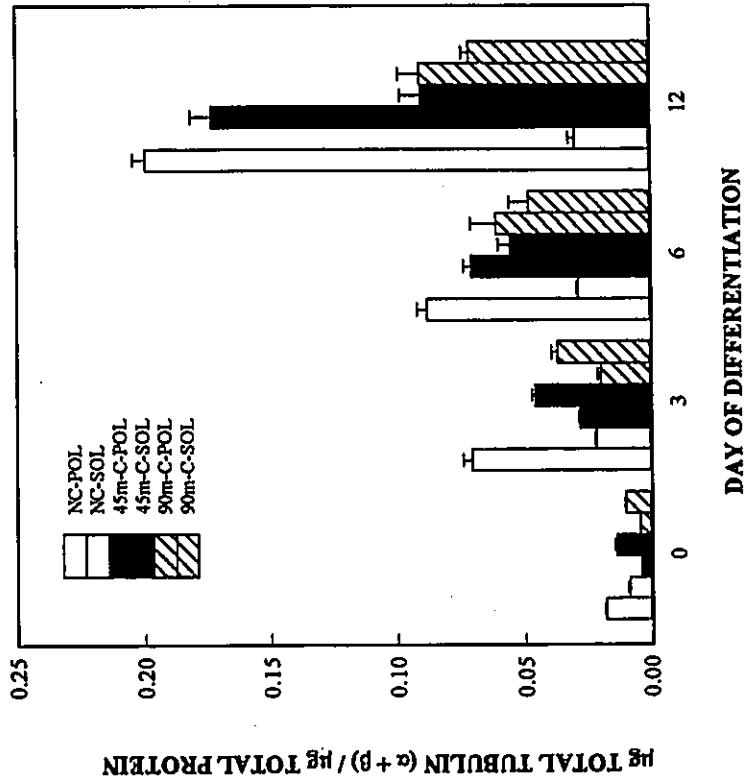
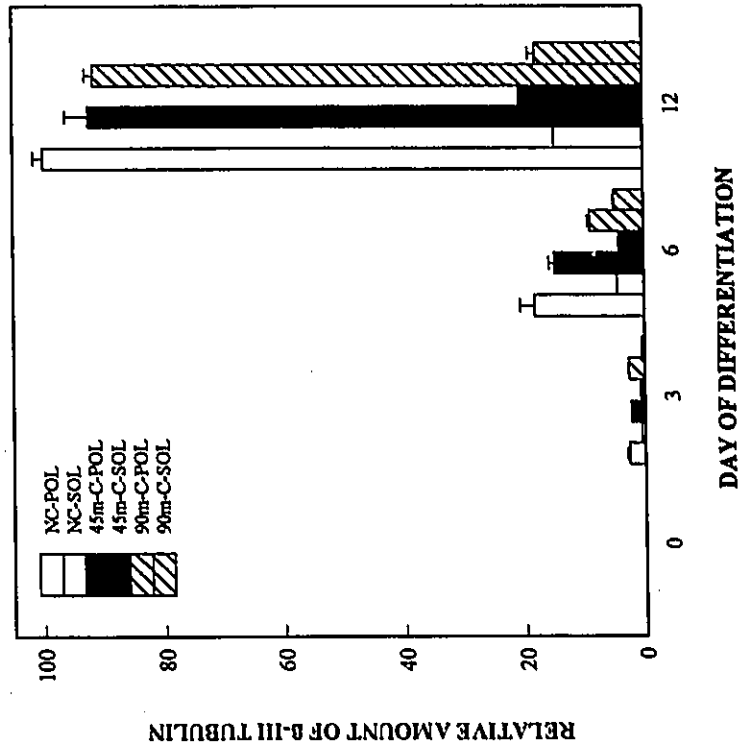


Figure 9. Analysis of absolute amounts of total tubulin, and relative amounts of β -III tubulin in soluble (Sol) and polymeric (Pol) tubulin fractions from non-colchicine treated (NC) and colchicine-treated (C) 45 and 90 min samples. Equal amounts of protein (1 μ g) from each fraction were analyzed by ELISA, using the anti- β -tubulin (DM1B) and anti- β -III tubulin antibody (TuJ1). β -III tubulin was expressed as a percentage of the maximum day 12 absorbance value. Each bar represents the mean of three analyses.

A



B



9B). In undifferentiated cells (day 0), total tubulin levels in untreated polymer and soluble fractions combined represented approximately 3% of total protein with few Mts resistant to colchicine. At day 3, an increase in total polymeric tubulin was noted, with less than half of polymeric tubulin resistant to 45 min colchicine treatment, and even less present following 90 min of colchicine. A further tubulin increase was evident in the polymeric fraction at day 6. At this time point relatively more of the polymeric tubulin was resistant to 45 min colchicine, with little further depolymerization after 90 min. By day 12, total tubulin levels in untreated polymer and soluble fractions combined represented almost 24% of total protein, with most of the polymeric fraction remaining following 45 min of colchicine. After 90 min of colchicine, however, total polymer tubulin levels were decreased by more than half.

Relative levels of β -III tubulin portrayed in Figure 9B exhibited a different pattern of distribution. Levels of β -III tubulin in day 0, 1 and 2 were too low to be detected by this procedure. At day 3, β -III tubulin was detected primarily in the polymer fraction, and appeared resistant to depolymerization by both 45 and 90 min colchicine treatments, but the levels were close to the limits of detection. Day 6 showed a distinct increase in β -III in polymer. Most of the β -III remained in polymer following 45 min of colchicine, but 90 min of colchicine resulted in a notable decrease in β -III in polymer. By day 12, a dramatic increase in the β -III polymer level was evident, and unlike total tubulin at this stage (Figure 9A), most of this β -

III polymer was resistant to both 45 and 90 min treatments of colchicine.

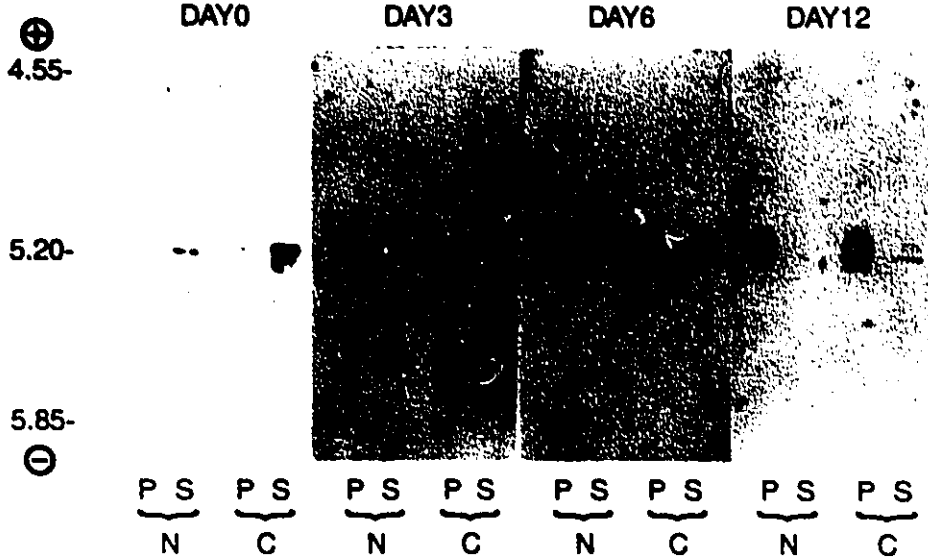
Extracts from three independent time-course experiments yielded identical trends for both total tubulin and β -III tubulin.

3. Class III β -Tubulin Isoforms Appear Concomitant with Increased Colchicine-Stability

The ELISA results showed that Mts were progressively stabilized during neuronal differentiation and that β -III tubulin was preferentially retained in the colchicine-stable polymer fraction. To determine if this correlated with the level of β -III posttranslational modification, polymer and soluble tubulin extracts were prepared from both colchicine-treated and non-colchicine treated cells and analyzed by IEF followed by immunoblotting with TuJ1. When an equal amount of protein (200 μ g) from each fraction was examined increasingly acidic β -III tubulin isoforms were detected in both polymer and soluble fractions (Figure 10). At day 0, one β -III tubulin isoform was detected at very low levels in primarily the soluble fraction of both colchicine-treated and untreated samples. This likely corresponds to the very low levels of unpolymerized β -III tubulin detected by immunofluorescence staining in a sub-population of P19 cells. By day 3, 3 isoforms were present in the polymeric fraction of untreated and colchicine-treated cells. The levels of β -III tubulin in the day 3 soluble fractions were too low (see Figure 9B) to detect any isoforms.

Figure 10. IEF followed by western blotting demonstrating β -III tubulin isoelectric variants present in colchicine-treated (C) and non-colchicine treated (N) polymer (P) and soluble (S) tubulin extracts prepared from undifferentiated (day 0) and RA-induced (days 3, 6, and 12) P19 cells. Equal amounts of total cell protein were loaded (200 μ g). Blot was probed with an anti- β -III tubulin antibody (TuJ1).

ISOELECTRIC VARIANTS OF β III-TUBULIN



* EQUAL AMOUNTS OF PROTEIN LOADED

In day 6 samples, at least 4 isoforms were detected in the polymer fraction of colchicine-treated and untreated cells. Three isoforms predominated in both corresponding soluble fractions. At least 2 more acidic isoforms appeared in the untreated and colchicine-treated polymer fraction by day 12.

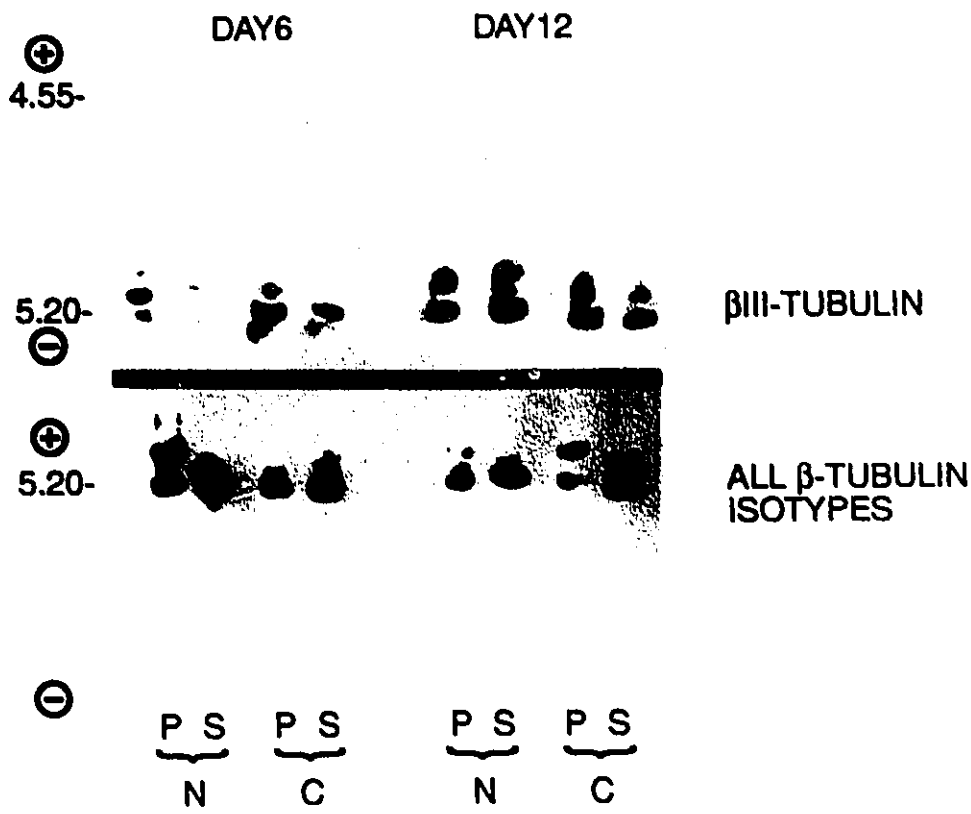
Since the amount of total tubulin increased dramatically following RA-induction (see Figure 9A), 200 μ g of total cell protein at each day of differentiation represented dramatically different amounts of tubulin. Therefore, to more accurately characterize the sorting of β -III tubulin isoforms between soluble and polymeric tubulin fractions, it was necessary to repeat the IEF study loading equivalent amounts (20 μ g) of total tubulin from each fraction as determined by ELISA (Figure 9A). Following immunoblotting with TuJ1 (Figure 11) 4 β -III isoforms were detected in the day 6 polymer fraction and 1 isoform in the soluble fraction of both colchicine-treated and untreated samples. By day 12, an increase in both the amount and the number of β -III isoforms was evident in all fractions.

Attempts to identify the posttranslational modifications of β -III tubulin in this culture system have met with limited success. To distinguish phosphorylated isoforms, 100-mm dishes of day 0, 3 and 6 cells were incubated overnight with $^{32}\text{PO}_4$. Whole cell extracts were then prepared, followed by immunoprecipitation with the TuJ1 antibody and isoforms of β -III tubulin present in the immunoprecipitate were subsequently separated by IEF. By day 6, at least one isoform of β -III tubulin was clearly phosphorylated (Figure 12). This isoform

was examined by phosphoamino acids analysis, and preliminary results suggested the site of this phosphorylation is a serine residue (data not shown).

To identify glutamylated β -III tubulin, whole cell P19 cell extracts collected at various days of neuronal differentiation were treated with either glutaminase or potato acid phosphatase prior to isoelectric focusing. Such treatments elicited no change in electrofocusing patterns (data not shown). Antibodies which recognize β -III tubulin isoforms are not yet available.

Figure 11. IEF followed by western blotting demonstrating β -III tubulin isoelectric variants present in colchicine-treated (C) and non-colchicine treated (N) polymer (P) and soluble (S) tubulin fractions prepared from day 6 and day 12 RA-induced P19 cells. Equivalent amounts of tubulin (20 μ g) from each fraction were loaded. Blot was first probed with an antibody to β -III tubulin (TuJ1) (upper frame). This blot was then stripped, and re-probed with an anti- β -tubulin antibody (DM1B) (lower frame) to confirm equal loading of tubulin.

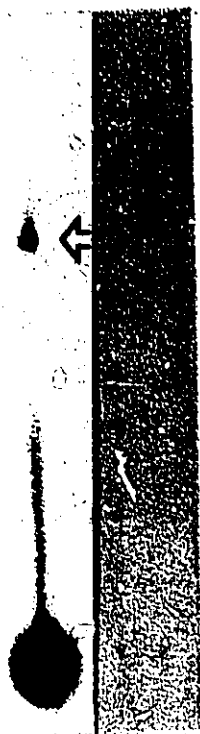


* EQUAL AMOUNTS OF TUBULIN LOADED

Figure 12. $^{32}\text{PO}_4$ -Labelling of Class III β -tubulin. $^{32}\text{PO}_4$ -labelled P19 cell extracts were immunoprecipitated with the TuJ1 antibody. This immunoprecipitate was then examined by isoelectric focusing followed by immunoblotting with TuJ1. Color development was with 4-chloro-1-naphthol. IEF immunoblots were aligned with autorads to reveal that at least one β -III isoform is phosphorylated at day 6. No $^{32}\text{PO}_4$ -labelling was evident at day 0 (undifferentiated P19 cells).

DAY 6

DAY 0



A



B

B. EFFECTS OF TAXOL ON CLASS III β -TUBULIN

1. Taxol Effects on Undifferentiated P19 Cells

Non-taxol treated undifferentiated P19 cells exhibited either typical interphase Mt arrays (Figure 13A') or normal mitotic arrangements with no β -III tubulin present in Mts (Figure 13A''). Following treatment with 1×10^{-9} M taxol, some Mt bundling was noted, but most cells maintained typical Mt arrays (Figure 13B'). Following treatment with 4×10^{-8} M taxol, bundling of Mts was more obvious, multiple asters could be detected in some cells (Figure 13 C') and β -III tubulin was incorporated into Mts, including aster configurations (Figure 13C''). Extensive Mt bundling was apparent and many cells contained multiple asters with a taxol treatment of 1×10^{-6} M (Figure 13D'). β -III tubulin was detected in Mts, including asters (Figure 13D'').

P19 cells treated with DMSO showed no unusual Mt rearrangements, and β -III tubulin was not detected in the Mts of these control cells (data not shown).

2. Effect of Taxol on P19 Neurons

At day 3, the P19 culture system typically consists of neurons, fibroblast-like and glial cells (reviewed by Falconer et al., 1994). Mts in typical interphase arrays, normal mitotic configurations and along unipolar neurites were labelled by the antibody YOL 1/34 (Figure 14B). Neuron-specific labelling with TuJ1 demonstrated the presence of polymerized β -III tubulin in Mts in the cell body and along the length of neuronal processes,

Figure 13. Effect of taxol on day 0 P19 cells. Hoechst staining of DNA (A-D) and double-immunofluorescence staining of Mts in undifferentiated P19 cells labelled using a monoclonal antibody to α -tubulin (A'-D') and a monoclonal antibody to β -III tubulin (A''-D''). No polymerized β -III tubulin is found in undifferentiated P19 cells (A''). Following 1×10^{-9} M taxol, Mt bundling (arrow) (B') is evident, and polymerized β -III tubulin is detected (B''). 4.2×10^{-8} M taxol results in Mt bundling or multiple aster formation (arrow) (C'). Polymerized β -III is present (C''). With 1×10^{-6} M taxol, many cells contain multiple asters or Mt bundles (arrow) (D') and β -III can be detected in aster configurations (D''). Bar, 10 μ m

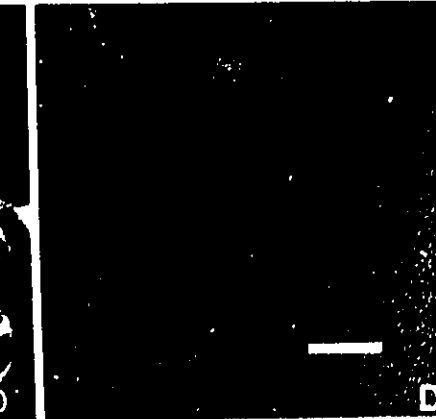
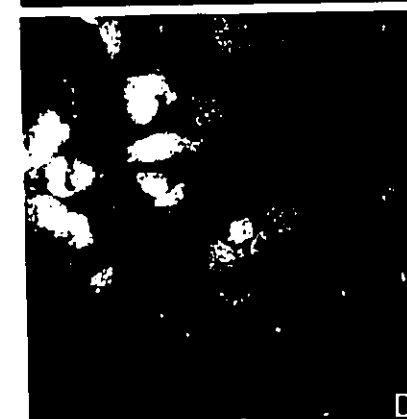
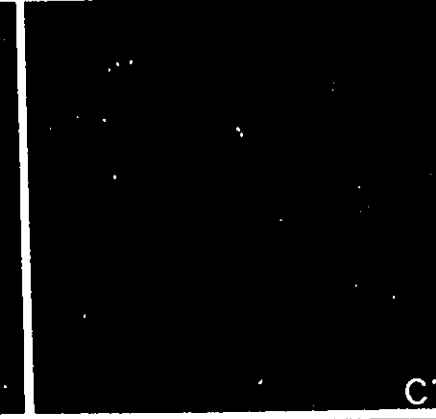
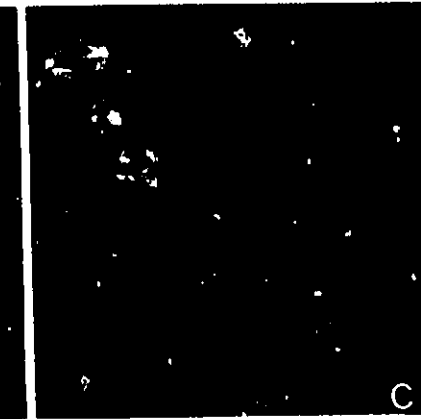
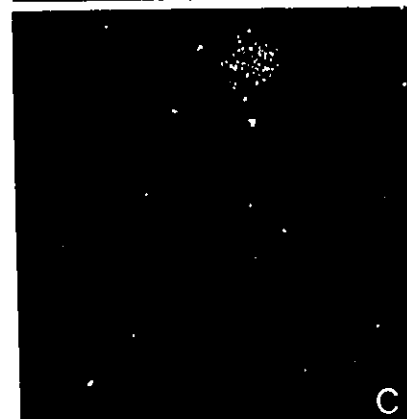
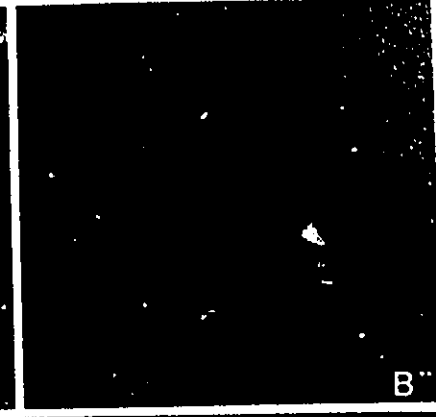
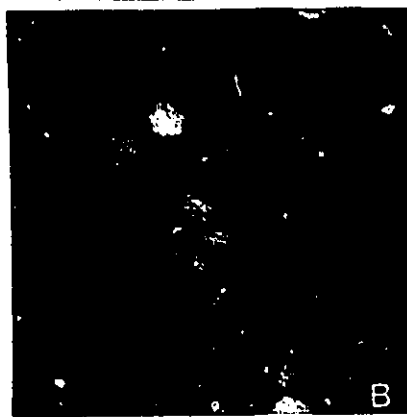
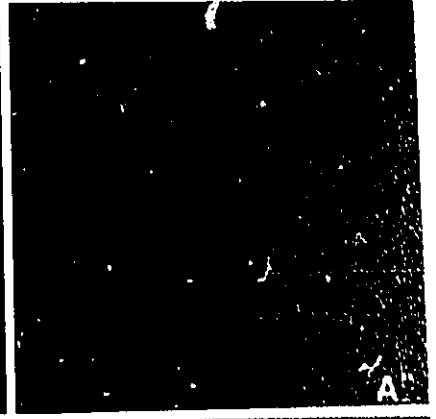
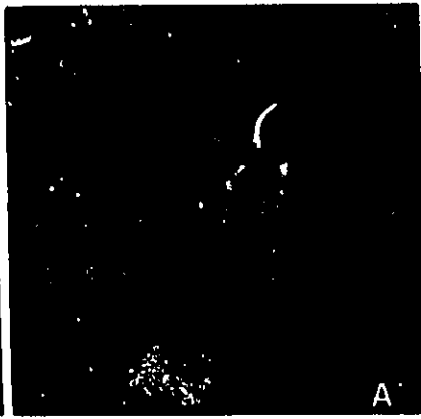
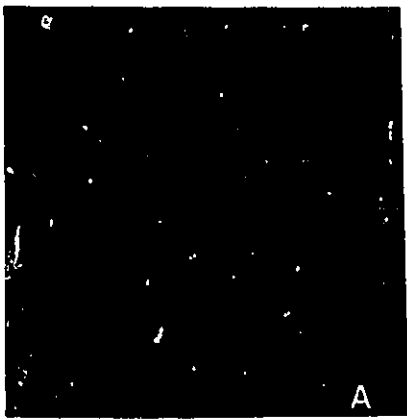
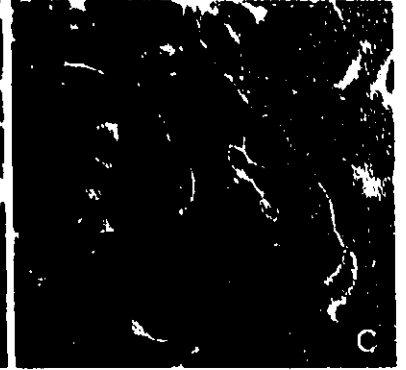
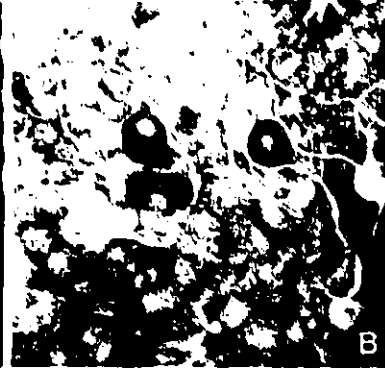
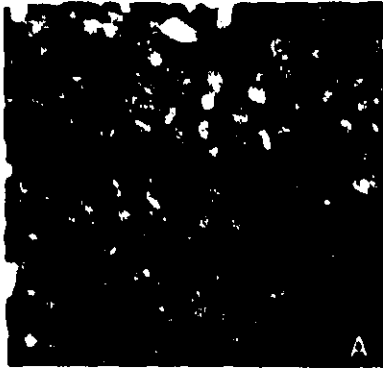


Figure 14. Mts present in day 3 P19 cells. Hoechst staining of DNA (A) and double-immunofluorescence staining of Mts in RA-induced P19 cells labelled using a monoclonal antibody to α -tubulin (B) and a monoclonal antibody to β -III tubulin (C). At day 3 many β -III positive cells have long neurite processes (C). Bar, 10 μ m



extending into typical growth cones (Figure 14C). RA-induced P19 cells treated with DMSO appeared as above (data not shown).

Taxol treatment of 1×10^{-9} M appeared to have little effect on neuronal Mts, and growth cones appeared normal. Neurons were abundant and displayed characteristic long, slender extensions (Figure 15A''). Following treatment with 1×10^{-8} M taxol, typical neurites appeared less abundant (Figure 15B''). Changes in growth cones were apparent and filopodia were present, but lamellipodia were less evident.

At 2.0×10^{-8} M taxol, extensive bundling of Mts was evident and some neurons exhibited short, very broad, neurite extensions (Figure 15C''). This phenotype was much more prevalent following treatment with 4×10^{-8} M taxol (Figure 15D''). The Mts in these broad neurites were intensely labelled by TuJ1 (Figure 15C'' and 15D''). Neurites exhibiting these short, broad extensions were uncommon at taxol concentrations greater than this.

Following treatment with 2×10^{-7} M (Figure 16A-A") and 1×10^{-6} M (Figure 16B-B") taxol, neurite-like processes were rare, and most neurons exhibited small cell bodies with occasional short, curved extensions intensely labelled by TuJ1. Growth cones were no longer apparent in neurons treated with 1×10^{-6} M taxol.

3. Polymer-Associated Posttranslational Modification of β -III tubulin

The immunofluorescence results demonstrated an apparent

Figure 15. Effect of taxol on P19 neurons. Hoechst staining of DNA (A-D) and double-immunofluorescence staining of Mts in day 3 RA-induced P19 cells treated with taxol for 24 hr. Cells were double-labelled using a monoclonal antibody to α -tubulin (A'-D') and a monoclonal antibody to β -III tubulin (A''-D''). Following 1×10^{-9} M taxol β -III tubulin is found along the length of apparently typical neurites (A''). With 1×10^{-8} M taxol fewer neurons have long extensions (B''). Following 2×10^{-8} M taxol neurites are obviously shorter and thicker at the base (C') and appear filled with polymerized β -III tubulin (arrow) (C''). At 4×10^{-8} M taxol, thick bundles of Mts appear at the site of neurite outgrowth (arrow) (D'').

Bar, 10 μ m

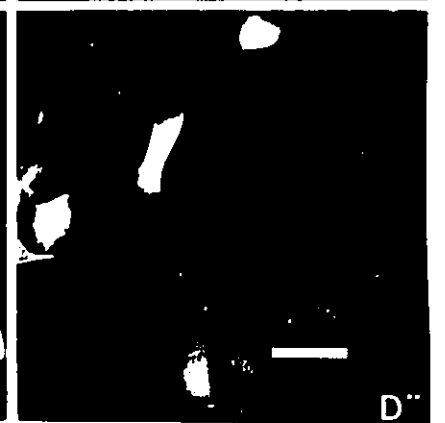
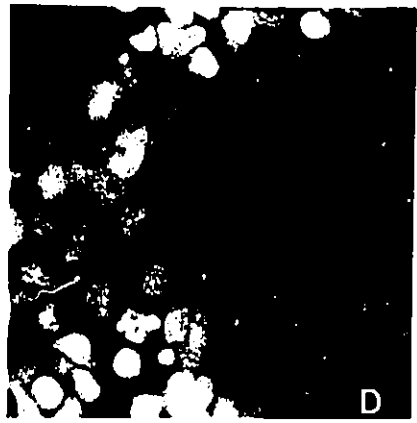
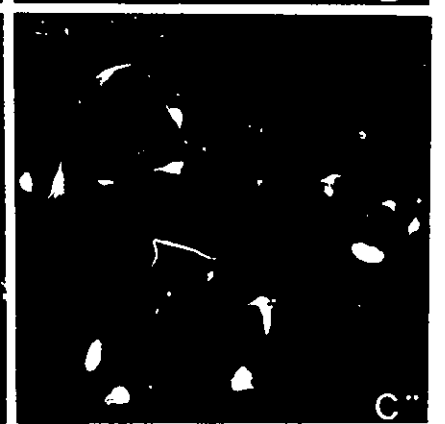
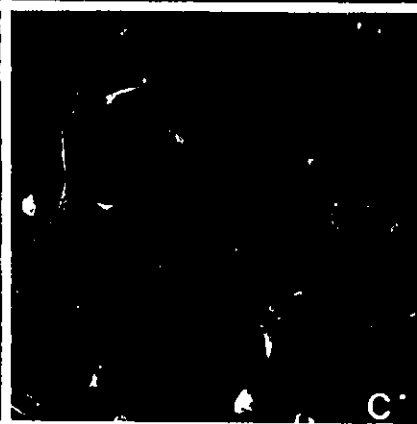
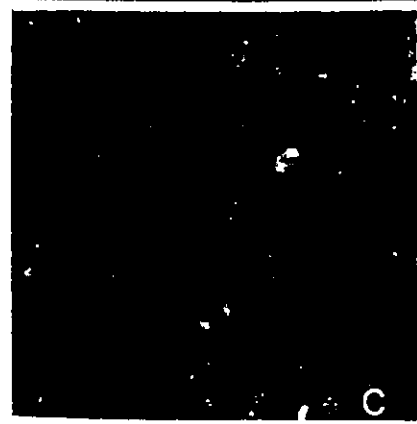
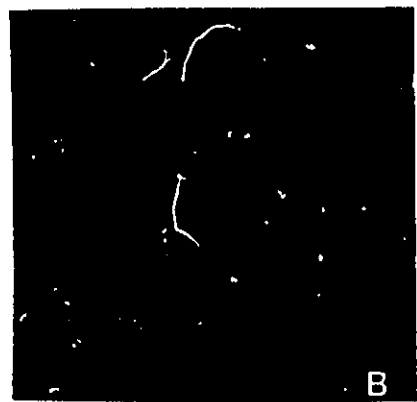
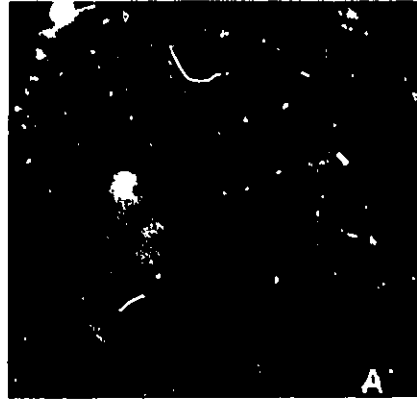
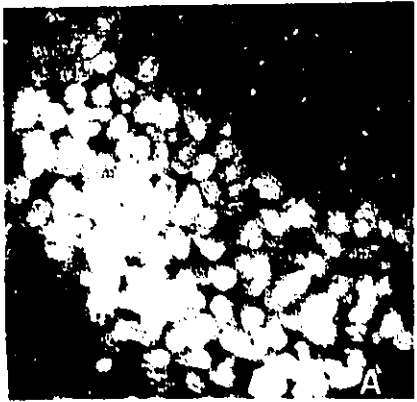
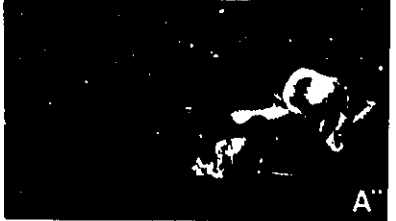
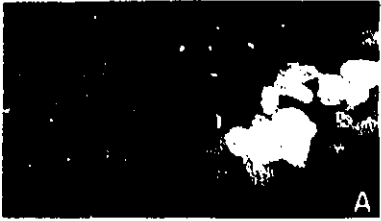


Figure 16. Effect of taxol on P19 neurons. Hoechst staining of DNA (A and B) and double-immunofluorescence staining of Mts in day 3 RA-induced P19 cells treated with taxol for 24 hr. Cells were double-labelled using a monoclonal antibody to α -tubulin (A' and B') and a monoclonal antibody to β -III tubulin (A'' and B''). Following 2×10^{-7} M (A' and A'') and 1×10^{-6} M taxol (B' and B''), neurites are either absent, or very short and convoluted. Bar, 10 μ m



increase in polymerized β -III tubulin following treatment with taxol. Previous studies (Section A), involving IEF followed by immunoblotting with TuJ1 demonstrated the appearance of increasingly acidic isoelectric variants of β -III tubulin, with 1 at day 0 (Figure 17, lane 1), 2 at day 3 (Figure 17, lane 2) and at least 6 present at day 12 (Figure 17, lane 3). To investigate the relationship between Mt assembly and posttranslational modification of β -III tubulin, the polymeric fraction of day 3 P19 neurons which had been treated with 10^{-6} M taxol for 24 hr was examined in a similar fashion. No alteration in the IEF pattern was observed in the DMSO control sample (compare lanes 2 and 4 in Figure 17). Treatment with taxol, however, resulted in the appearance of an additional more acidic isoform of β -III tubulin (Figure 17, lane 5).

Figure 17. Effect of taxol on β -III tubulin. IEF followed by western blotting demonstrating β -III tubulin isoelectric variants present in polymer tubulin extracts prepared from non-taxol treated undifferentiated (1), day 3 (2), and day 12 (3) P19 cells and day 3 P19 cell DMSO control (4) and following treatment with 1×10^{-6} M taxol (5). Equal amounts of total cell protein were loaded (200 μ g). Blot was probed with an anti- β -III tubulin antibody (TuJ1). (+) refers to H⁺ and (-) refers to OH⁻.

+



-

1

2

3

4

5

C. EXPRESSION OF CLASS III β -TUBULIN IN UNDIFFERENTIATED P19 CELLS

1. Assembly of Class III β -tubulin Transiently Expressed in Undifferentiated P19 Cells

The ELISA (Figure 9) and IEF (Figure 10) results showed a correlation between the expression and extent of posttranslational modification of β -III tubulin with increased colchicine stability of Mts in differentiating neurons. These changes in β -III tubulin are occurring concurrently with alterations in, and induction of, expression of other tubulin isotypes and of MAPs (Falconer et al., 1994). To test the effects of increased β -III tubulin expression in the absence of these other changes undifferentiated P19 cells were transfected with the *Pgk-1- β -III* mammalian expression vector. β -III tubulin was detected in both mitotic spindles and in interphase Mts of transfected cells (Figure 18a'-a''). Careful examination by epifluorescence microscopy revealed that all Mts present in transfected cells appeared to be labelled by both the YOL 1/34 and TuJ1 antibodies. Thus, β -III tubulin is assembly competent in non-neuronal cells and appears to be indiscriminately incorporated into all Mts. In most transfected cells observed the expression of β -III tubulin had no apparent effect on Mt organization, although some cells exhibited bizarre Mt organizations (data not shown). Results similar to those described above were obtained following transfection with the *RSV- β -III* expression vector, although levels of β -III tubulin appeared lower by immunofluorescence microscopy.

Figure 18. Transient transfection with wild-type β -III tubulin. Phase contrast (a-d) and immunofluorescence micrographs of Mts in P19 cells transiently transfected with wild-type *Pgk-1- β -III* tubulin following treatment with colchicine. Cells were double-labelled using a monoclonal antibody to α -tubulin (a'-d') and a monoclonal antibody to β -III tubulin (a''-d''). Typical Mt arrays are present in untreated P19 cells (a'), with transfected cells exhibiting β -III tubulin in interphase, mitotic spindle and midbody Mts (a''). A decrease in total Mts is apparent following 15 (b') and 30 (c') min of colchicine, with a corresponding decrease in β -III Mts (b'') and (c''). Very few Mts are resistant to depolymerization following 45 min colchicine-treatment (d') including those which have incorporated β -III tubulin (d''). Bar, 10, μ m.

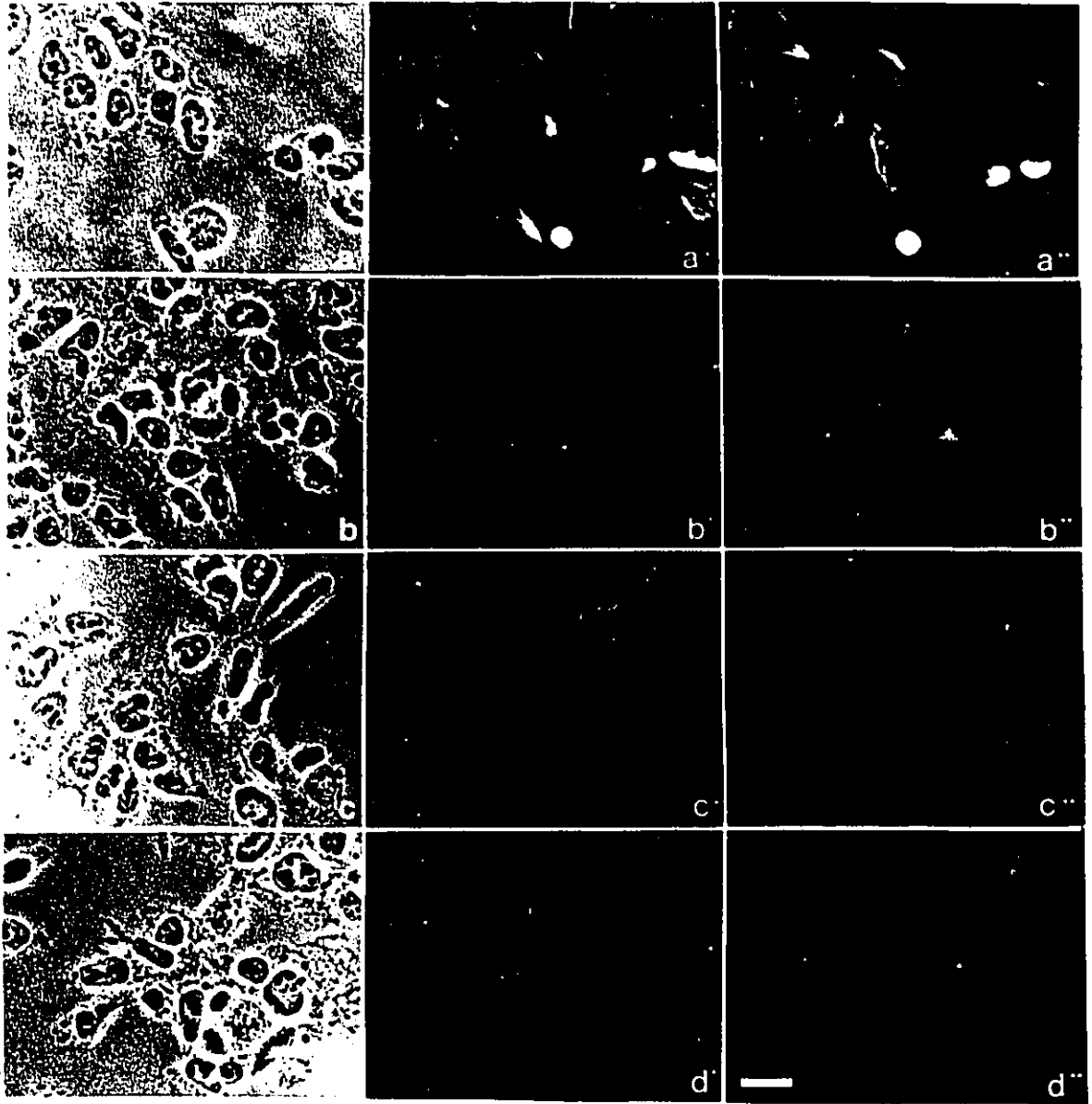
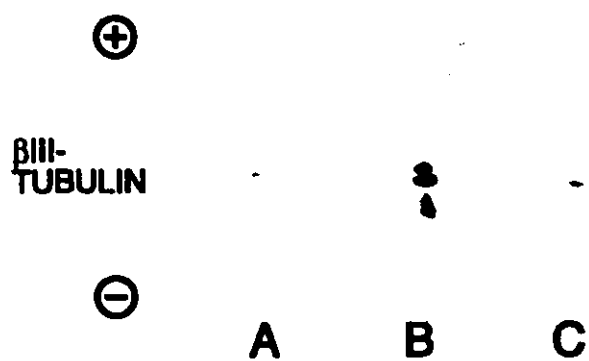


Figure 19. IEF following transfection with wild-type β -III tubulin. IEF followed by immunoblotting with TuJ1, demonstrating β -III tubulin isoelectric variants present in the polymer fraction of P19 cells transfected with wild-type *Pgk-1*- β -III tubulin. Control (A) transfection with no *Pgk-1*- β -III added. Transfected β -III tubulin undergoes at least one posttranslational modification (B). Neither β -III tubulin electrovariant is resistant to depolymerization following 45 min colchicine treatment (C).

**IEF OF POLYMERIC FRACTIONS OF
P19 EC CELLS TRANSFECTED WITH
PGK- β III**



2. Colchicine-Stability of Transfected β -III tubulin

To determine if the expression of β -III tubulin in the undifferentiated P19 cells altered Mt stability colchicine was added to the medium of transfected cells and samples were prepared for double-immunofluorescence labelling at intervals. No difference between the colchicine-stability of the β -III positive Mts in the transfected cells and the Mts in adjacent non-transfected P19 cells was noted. Following 15 min treatment with colchicine some Mts remained (Figure 18b' and b''), fewer still were evident at 30 min (Figure 18c' and c''), and after 45 min all Mts were disassembled in most cells (Figure 18d' and d'').

IEF and immunoblotting were used to investigate the posttranslational state of transfected β -III tubulin. Two β -III tubulin isoelectric variants were evident in the polymer fraction from transfected P19 cells (Figure 19). Consistent with the immunofluorescence results (Figure 18), the analysis also showed that the β -III-containing Mts were colchicine labile (Figure 19). While β -III tubulin undergoes at least one posttranslational modification in non-neuronal P19 cells, expression of β -III tubulin and this modification are insufficient to confer colchicine-stability to Mts in these cells.

3. Assembly and Colchicine-Stability of Ala⁴⁴⁴ and Glu⁴⁴⁴ β -III Tubulin in Undifferentiated P19 Cells

To investigate a potential role for Ser⁴⁴⁴ phosphorylation

of Class III β -tubulin, undifferentiated P19 cells were transiently transfected with β -III tubulin mutant expression vectors. One β -III tubulin mutant cannot undergo Ser⁴⁴⁴ phosphorylation (β -IIIAla⁴⁴⁴), and the other carries a negative charge at that residue (β -III Glu⁴⁴⁴) to mimic a constitutively phosphorylated β -III tubulin. Following transfection, P19 cells were double-immunolabelled with TuJ1 and YOL 1/34 and the cells were carefully examined by epifluorescence microscopy to assess the assembly competency and localization of the transfected tubulin. Both the β -IIIAla⁴⁴⁴ (Figure 20a'-a'') and the β -III Glu⁴⁴⁴ (Figure 21a'-a'') mutants were detected in all mitotic spindles and interphase Mts present in transfected cells. Mt morphology appeared typical of undifferentiated P19 cells, although as with the wild-type β -III tubulin expression, a few cells with bizarre Mt arrangements were noted. The Mts containing mutant β -III tubulin were indistinguishable from the Mts containing wild-type β -III (Figure 19a'-a'').

To assess the colchicine stability of the Mts containing mutant β -III tubulin in the undifferentiated P19 cells, colchicine was added to the medium of transfected cells and samples were prepared for double-immunofluorescence labelling at intervals. No difference between the colchicine-stability of the Mts containing mutant β -III tubulin was noted in transfected cells and the Mts in adjacent non-transfected P19 cells was noted. Following 15 min treatment with colchicine some β -IIIAla⁴⁴⁴ Mts (Figure 20b' and b'') remained. Fewer were evident at 30 min (Figure 20c' and c''), and after 45 min virtually all

Mts were disassembled in most cells (Figure 20d' and d''). Similarly, some β -IIIIGlu⁴⁴⁴ Mts (Figure 21c' and c'') remained after 15 min colchicine, with fewer evident at 30 min (Figure 21c' and c''), and all Mts in most cells disassembled after 45 min (Figure 21d' and d'').

Figure 20. Phase contrast (a-d) and immunofluorescence micrographs of Mts in P19 cells transiently transfected with *Pgk-1- β -IIIAla⁴⁴* tubulin following treatment with colchicine. Cells were double-labelled using a monoclonal antibody to α -tubulin (a'-d') and a monoclonal antibody to β -III tubulin (a''-d''). Typical Mt arrays are present in untreated P19 cells (a'), with transfected cells exhibiting β -III tubulin in interphase and mitotic Mts (a''). A decrease in total Mts is apparent following 15 (b') and 30 (c') min of colchicine, with a corresponding decrease in Mts containing mutant β -III tubulin (b'') and (c''). Very few Mts are resistant to depolymerization following 45 min colchicine-treatment (d') including those which have incorporated the mutant β -III tubulin (d''). Bar, 10, μ m.

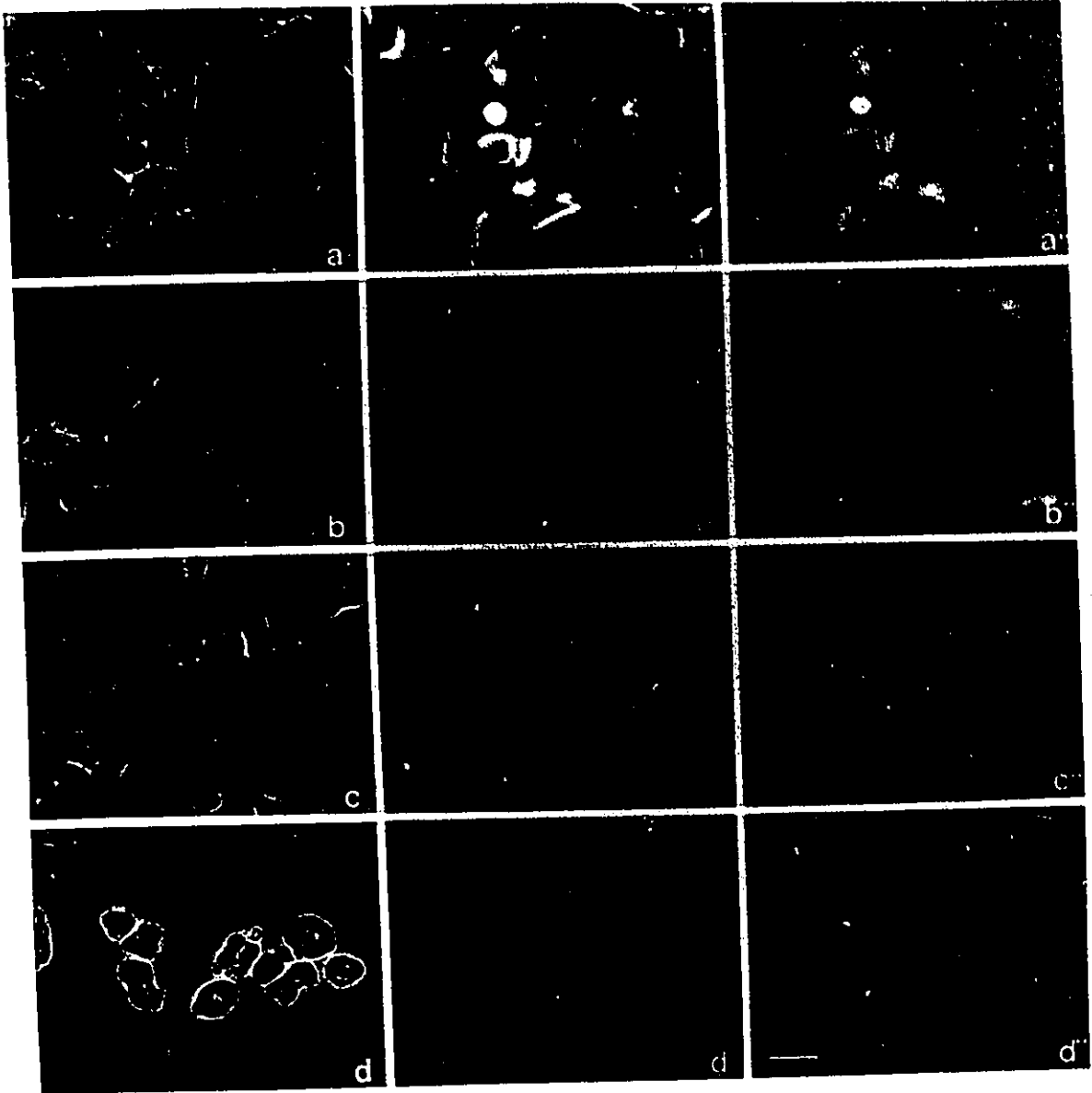
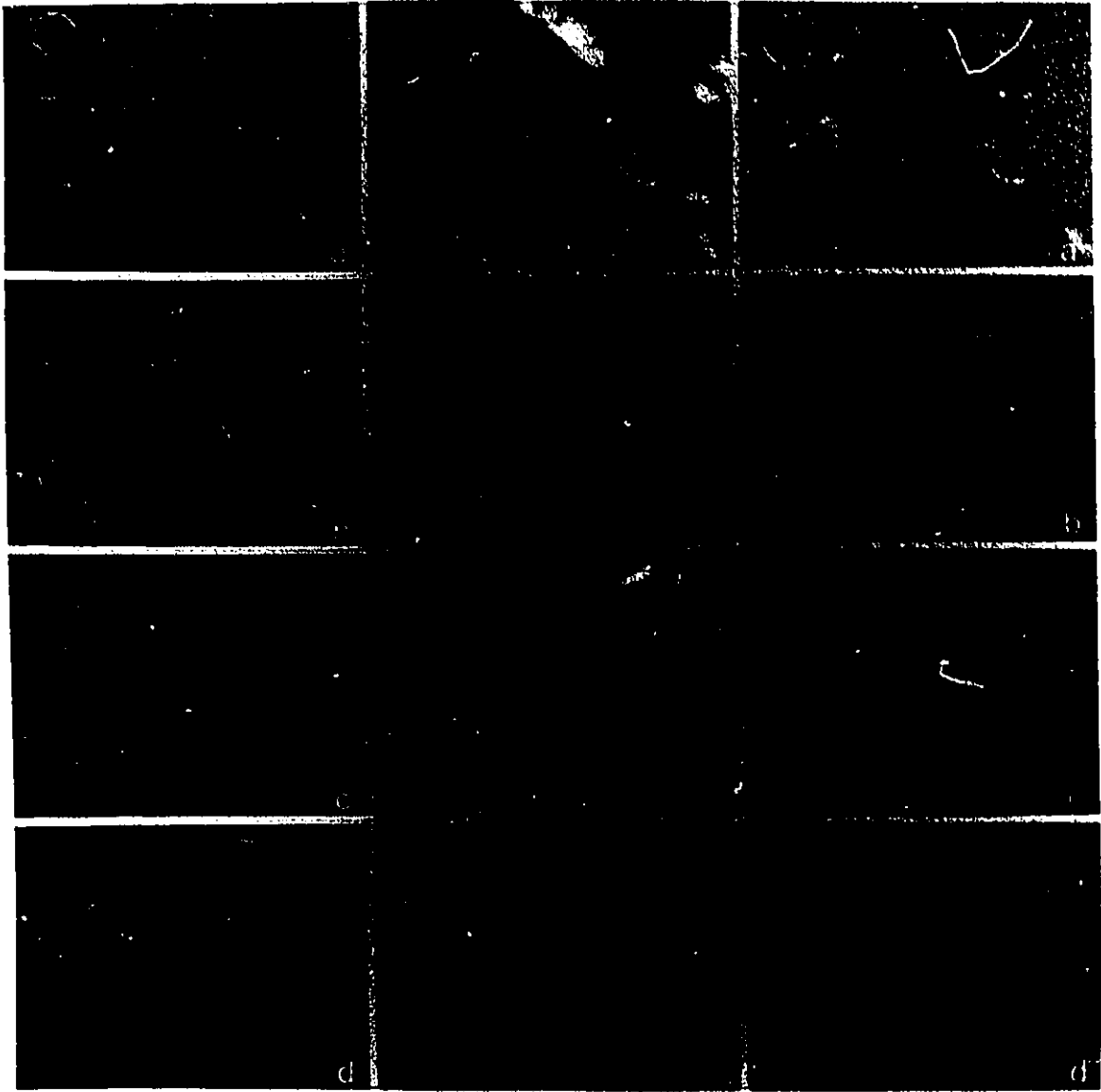


Figure 21. Phase contrast (a-d) and immunofluorescence micrographs of Mts in P19 cells transiently transfected with *Pgk-1- β -III*Glu⁴⁴ tubulin following treatment with colchicine. Cells were double-labelled using a monoclonal antibody to α -tubulin (a'-d') and a monoclonal antibody to β -III tubulin (a''-d''). Typical Mt arrays are present in untreated P19 cells (a'), with transfected cells exhibiting β -III tubulin in interphase Mts (a''). A decrease in total Mts is apparent following 15 (b') and 30 (c') min of colchicine, with a corresponding decrease in Mts containing mutant β -III tubulin (b'') and (c''). Very few Mts are resistant to depolymerization following 45 min colchicine-treatment (d') including those which have incorporated the mutant β -III tubulin (d''). Bar, 10, μ m.



DISCUSSION

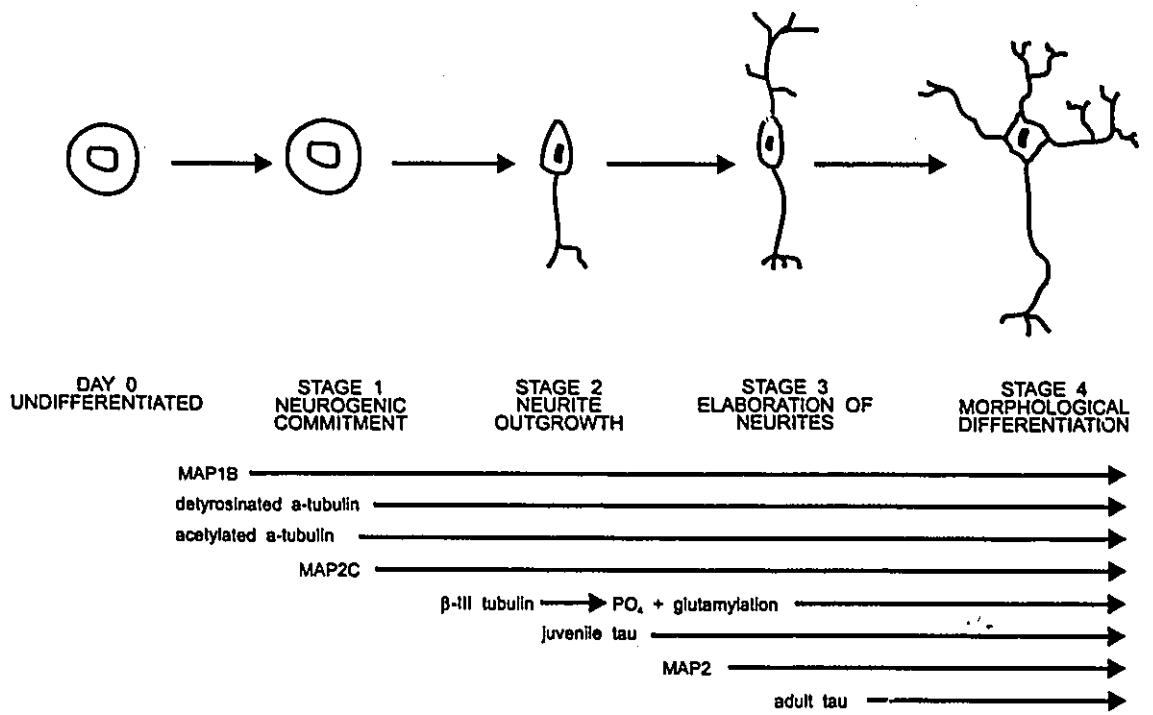
EXPRESSION OF CLASS III β -TUBULIN IN P19 NEURONS

In previous studies using RA-induced P19 cells the developmentally-regulated expression of MAPs and tubulins was correlated with increasing stability to colchicine, providing a profile very similar to that seen in developing brain (Figure 22) (see Falconer et al., 1994 for review). MAP1B is present in undifferentiated P19 cells and is the first MAP to be up-regulated following the addition of RA. MAP2c appears at day 2, juvenile tau at day 3, followed by MAP2 at day 4 and adult tau at day 6 (Falconer et al., 1989a, 1992, 1994).

Most Mts present in undifferentiated P19 cells exhibit low levels of acetylated and detyrosinated tubulin and are depolymerized in the presence of 1 μ g/mL colchicine for 45 min. Within the first 24 hr of differentiation acetylated and detyrosinated tubulin levels rise and Mts show increased stability to colchicine (Falconer et al., 1989a,b). By 2 days of differentiation Class II β -tubulin expression is up-regulated and Class III β -tubulin is detected in a subset of cells. At this time, there is a preferential sorting of Class II β -tubulin into colchicine-stable Mts and an apparent exclusion of Class III β -tubulin (Falconer et al., 1992). The exclusion of β -III tubulin from stable Mt arrays has also been seen during early stages of differentiation of hippocampal pyramidal neurons and cerebellar macroneurons (Ferreira and Caceres, 1992).

Low levels of β -III tubulin are expressed in some cells in

Figure 22. Summary of MAP and tubulin expression in RA-induced P19 cells.



cultures of undifferentiated P19 cells, but it is rarely found incorporated into the Mts of these cells. Soluble β -III tubulin can be detected by immunofluorescence microscopy when these cells are fixed in -20°C methanol (data not shown). The early increase in expression of β -III tubulin mRNA and the subsequent appearance of β -III tubulin in the mitotic spindle of neuroblasts seen in this study suggests a developmental role which precedes the physical requirement for additional tubulin subunits to facilitate neurite outgrowth. Consistent with our previous observations (Falconer et al., 1992), β -III tubulin appears susceptible to colchicine-induced depolymerization prior to neurite outgrowth. By day 3, however, neurite outgrowth is evident and β -III tubulin is detected in colchicine-resistant Mts in developing neurons. Increases in Class III β -tubulin protein and mRNA levels occur concurrently until approximately day 7, when β -III mRNA levels begin to decrease while protein levels remain high, suggesting that β -III tubulin is stabilized at later stages of development. A similar suggestion has been made for the cerebral cortex where β -III mRNA levels were reported to decrease more dramatically than protein levels during development (Jiang and Oblinger, 1992). The ELISA results showing that most β -III tubulin remains associated with the colchicine-stable polymer fraction at later stages of differentiation support this suggestion.

Although it was clear that β -III tubulin was included in the colchicine-stable Mts of day 6 and 12 neurons, the presence of β -III tubulin-containing aggregates which remained associated

with the stable cytoskeletal fraction following nonionic detergent extraction was also noted. The form of this β -III tubulin was difficult to resolve by epifluorescence or confocal microscopy. Anomalous labelling of β -III tubulin, including the distinctly "patchy" distribution of β -III tubulin along the Mts of PC-12 cells (Joshi and Cleveland, 1989) and the presence of detergent-resistant, nonfilamentous β -III in the soma of PC-12 cells (Asai and Remolona, 1989) has been reported. β -III-containing aggregates were not observed in non-colchicine treated cells. The aggregates in treated cells may represent an accumulation of β -III tubulin which was being translocated or non-Mt oligomers of β -III tubulin resulting from disassembly.

In addition to showing the increases in colchicine-stable Mts during neuronal differentiation, ELISA also showed that there are large increases in total tubulin and in β -III tubulin during differentiation. As in 3T3 fibroblasts, in which tubulin is 3.5% of total protein (Anderson, 1979), total tubulin is approximately 3% of total protein in undifferentiated P19 cells. By day 12 of differentiation tubulin has increased to 24% of total protein and exceeds the value of 13.5% of total protein for mouse brain (Anderson, 1979). This is consistent with immunofluorescence observations that the differentiated P19 cultures consist primarily of neurons and those of McBurney et al. (1988) showing that up 85% of the cells express neuronal markers by day 6.

Although ELISA and immunoblotting of IEF gels showed large increases in β -III tubulin in the polymer fractions at days 6

and 12, the immunofluorescence staining with the TuJ1 antibody appeared lower than in day 3 neurons. The cause of this reduction in staining is unknown, but association of other proteins (e.g. MAPs) and/or the posttranslational modifications of β -III tubulin shown in this study may mask or alter the epitope recognized by the antibody in the microscopy samples.

The developmental appearance of β -III tubulin isoforms detected by IEF, from 1 at day 0 to at least 6 at day 12, is similar to that described in rat brain (Lee et al., 1990b). Antibodies are not available to definitively identify the modification represented by each isoform. The first band detected on IEF immunoblots is presumably the unmodified gene product, and preliminary studies suggest that the first β -III isoform to appear is the result of serine phosphorylation as has been reported in neuroblastoma cells (Gard and Kirschner, 1985; Eddé et al., 1989). Subsequent electrovariants are likely glutamylated isoforms of β -III tubulin as more than 85% of β -III tubulin is glutamylated in adult brain (Eddé et al., 1991, Alexander et al., 1991).

The appearance of these β -III isoforms occurs concomitantly with an increase in colchicine stability of Mts containing β -III tubulin suggesting that posttranslational modifications of β -III tubulin play a role in regulating Mt stability during neuronal differentiation. This temporal change in Mt dynamics is likely to also involve enhanced interactions with developmentally regulated MAPs. The reversible (Audebert et al., 1993) and developmentally regulated (Audebert et al., 1994) addition of up

to six glutamate residues to the glutamate γ -carboxyl group of β -tubulin can result in the formation of a branched and negatively charged polypeptide tail which may modulate MAP interaction.

Boucher et al., (1994) have shown that both MAP2 and tau associate preferentially with moderately glutamylated α - and β -tubulin isoforms while exhibiting only minimal binding to both the unmodified and more highly modified tubulin isotypes. The length of the polyglutamylated tail appears to modulate MAP binding indirectly through resultant conformational changes which render the MAP binding region inaccessible. The increased levels of moderately glutamylated β -III tubulin isoforms detected in the colchicine-stable Mts of day 6 P19 neurons (Figures 10 & 11) are coincident with the expression of MAP2 and adult tau (Figure 22) (Falconer et al., 1994). The enzymes responsible for tubulin posttranslational modifications may be co-regulated with MAP expression to permit enhanced tubulin/MAP interactions.

Other posttranslational modifications of tubulin, including acetylation, and detyrosination occur preferentially on tubulin in polymerized Mts. Following disassembly of these modified tubulin subunits, acetylated tubulin is rapidly de-acetylated and detyrosinated tubulin is promptly re-tyrosinated (Bulinski and Gundersen, 1991). In contrast, in this study isoforms of β -III tubulin were detected in both the soluble and the polymer fractions of differentiated P19 neurons. Audebert et al., (1993) have also detected the presence of polymer and soluble

glutamylated α - and β -tubulin isoforms in differentiating mouse brain. Their results suggest that like acetylation and detyrosination, glutamylation of β -tubulin occurs on polymerized tubulin and deglutamylation occurs on soluble tubulin. They report that deglutamylation of the 4-6th unit of soluble polyglutamylated α -tubulin occurs readily, while units 1-3 are more resistant. The kinetics of this deglutamylation may serve to maintain a soluble pool of tubulin which is optimally modified to interact with MAPs (Boucher et al., 1994).

Another factor which may affect the presence of β -III tubulin in stable Mts is the increased expression of β -II tubulin during neuronal differentiation (Falconer et al., 1992; Jiang and Oblinger, 1992; Oblinger and Kost, 1994). Tubulin isotype composition has been shown to alter the assembly kinetics of Mts *in vitro*. For example, the divergent erythrocyte Class VI $\alpha\beta$ -tubulin forms Mts which are more stable, and less affected by changes in ionic strength and pH than Mts assembled from a mixture of brain β -tubulin isotypes (Classes α I, α II, α III and α IV). By combining varying amounts of erythrocyte $\alpha\beta$ -tubulin with brain $\alpha\beta$ -tubulin isotypes, it is possible to predictably alter the assembly properties of the resultant tubulin mix. In addition, isotype gradients are evident along the length of the resultant Mt copolymers (Rothwell et al., 1986; Baker et al., 1990).

Mts made from purified $\alpha\beta$ III are more dynamic than Mts made from unfractionated tubulin containing all brain isotypes, purified $\alpha\beta$ II or $\alpha\beta$ IV tubulin dimers. A mixture of 20% $\alpha\beta$ III

and 80% $\alpha\beta$ II tubulin, however, results in Mts which are less dynamic than Mts made from pure $\alpha\beta$ II tubulin (Panda et al., 1994). Class II β -tubulin represents 58%, and Class III β -tubulin 25% of the β -tubulin in bovine brain preparations (Banerjee et al., 1988). This tubulin isotype ratio may be optimized to contribute to the temporal increase in Mt stability noted in differentiating neurons.

The results of this study document a progressive increase in Mt stability and reveal a highly regulated pattern of β -III tubulin expression and posttranslational modification in P19 neurons. Some of the molecular mechanisms underlying this increased stability have been examined in the remainder of this thesis.

EFFECTS OF TAXOL ON P19 CELLS

In undifferentiated P19 cells, as in other cells, taxol-treatment results in either extensive Mt bundling or multiple aster formation, depending on the cell cycle stage. In interphase cells, taxol induces the spontaneous assembly of stable Mts in a time and concentration-dependent manner (Horwitz et al., 1986a). This results in characteristic Mt bundles which are generally not associated with the microtubule-organizing center (DeBrabander et al., 1981). The taxol-induced Mt bundles are not irreversibly stabilized and are disassembled and replaced by multiple asters as cells enter mitosis (DeBrabander et al., 1981; Roberts et al., 1989; Verde et al., 1991).

Taxol arrests HeLa cells and mouse fibroblasts in the G₂ and

M-phases of the cell cycle, while not interfering with S phase DNA synthesis (Horwitz et al., 1986b). This was apparent from the Hoechst staining which showed the larger nuclei (Figure 14 B,C,D) indicative of the polyploid nature of many of the taxol-treated cells. Additionally, a taxol dose-related increase in cell death was apparent and collapse and condensation of DNA and fragmentation of the nuclei can be seen (Figure 13 C). These changes are typical of cells undergoing apoptosis in response to a variety of treatments, including taxol (Bhalla et al., 1993).

The effect of taxol on the initiation and outgrowth of neurites has been examined in primary cultures of dorsal root ganglia (Letourneau and Ressler, 1984; Letourneau et al., 1987). A treatment with 7×10^{-9} M taxol for 24 hr was the lowest dose reported to stimulate Mt assembly (Letourneau and Ressler, 1984). No changes in the neurites treated with the 1×10^{-9} M taxol were noted, but an apparent shortening of neurite length was observed when differentiating neurons were exposed to a taxol concentration of 1×10^{-8} M. At slightly higher taxol concentrations, neurites were consistently shorter and much broader, with little branching.

Low levels of taxol do not appear to prevent neurite initiation, but neurite elongation is affected. Neurite elongation is likely inhibited by the impact of the extensive bundling and/or stabilization of Mts on the growth cone (Letourneau and Ressler, 1984). It is known that the highly dynamic Mts which normally extend into the lamellipodium of the growth cone are important in neurite extension (Tanaka and

Kirschner, 1991; Tanaka and Kirschner, 1995; Tanaka et al., 1995).

In untreated day 2 P19 neurons, β -III tubulin is preferentially found in soluble, unpolymerized form (Falconer et al., 1992). However, the broad neurites of taxol-treated neurons appear filled with Mts containing β -III tubulin (Figure 15 D''). Similarly, although soluble β -III tubulin is present in some undifferentiated P19 cells, treatment of day 0 P19 cells with 4×10^{-8} M or more taxol altered the normal subcellular sorting of tubulin by promoting the polymerization of β -III tubulin. Asai and Remolona (1989) treated PC-12 cells with taxol to induce polymerization of β -III tubulin which appeared to be largely excluded from Mt polymer. These results demonstrate that the β -III tubulin present in the soluble fraction of these cells is assembly competent and molecular mechanisms must exist which regulate the assembly of β -III tubulin into Mts. C-terminus isotype differences and posttranslational modifications may be involved as β -III tubulin has been shown to have distinctive *in vitro* assembly properties when compared to other mammalian β -tubulins. Such differences may also influence MAP-directed polymerization (Banerjee et al., 1990; reviewed by Ludueña, 1993; Panda et al., 1994).

Taxol-treated day 3 neurons display a third β -III tubulin isoform suggesting that this posttranslational modification is associated with increased assembly of β -III into Mts. Taxol has been reported to stimulate phosphorylation of β -III tubulin in undifferentiated and differentiated neuroblastoma cells (Gard

and Kirschner, 1985). In a preliminary study, when day 2 RA-induced P19 cells were incubated for 24 hr in the continued presence of $^{32}\text{PO}_4$ and taxol, the taxol-induced isoform was not labelled (data not shown). The levels of β -III tubulin are low at day 3 in this culture system, and it is difficult to detect phosphorylation of β -III tubulin in non-taxol treated day 3 cultures. It is possible that following treatment with taxol, the levels of phosphorylated β -III tubulin present are too low to permit detection, although this seems unlikely. In early differentiating mouse brain neurons taxol stimulates glutamylation of β -III tubulin (Audebert et al., 1993) and the taxol-induced β -III tubulin isoform noted in the P19 culture may, therefore be the result of glutamylation. The enzyme(s) responsible, must be present in these early neurons, but its activity may be limited by the amount of β -III tubulin present in Mt polymer. The accumulation of increasingly acidic isoforms of β -III tubulin at later times of differentiation may be a consequence of the increased assembly of β -III tubulin into stable Mts.

EXPRESSION OF WILD-TYPE AND TWO Ser⁴⁴ MUTANT β -III TUBULINS IN P19 CELLS

Following transient transfection of undifferentiated P19 cells with the wild-type *Pgk-1- β -III tubulin* construct distinct populations of cells with robust β -III tubulin labelling of Mts were evident by immunofluorescence microscopy. As in other tubulin transfection experiments (Bond et al., 1986; Joshi et

al., 1987; Lewis et al., 1987; Lopata and Cleveland, 1987) the transiently expressed β -III tubulin was capable of co-assembling along with the other tubulin isotypes present *in vivo* into typical Mt arrays. Most β -III transfected cells exhibited apparently normal Mt arrays, although some cells displayed bizarre morphologies that may be indicative of a toxic effect. Similar results were observed following transfection of P19 cells with each of the mutant β -III tubulin expression vectors: *Pgk-1- β -IIIAla⁴⁴⁴* and *Pgk-1- β -IIIIGlu⁴⁴⁴*.

While greater than 10 to 20% overexpression of β -tubulin has been shown to be lethal in higher eukaryotic cells, a 'threshold effect' has been described in which a minimal level of tubulin expression is necessary before any unique isotype properties can be assessed *in vivo* (reviewed by Murphy, 1991). In *Drosophila*, when the minor β 3 tubulin isotype was co-expressed with the major β 2 tubulin isotype, disruption of axoneme assembly resulting in male sterility was noted only when β 3 levels exceeded 20% of the total tubulin pool. Flies expressing β 3 levels lower than this were normal (Hoyle and Raff, 1990; Raff, 1994). One limitation of tubulin transfection studies, therefore, is attaining sufficiently high levels of tubulin expression without killing the cells.

The *Pgk-1* promoter employed here is constitutively active in P19 cells and has been demonstrated to yield high transcription levels in these cells (reviewed by McBurney, 1993). However, attempts to isolate permanently transfected clones expressing wild-type β -III tubulin were unsuccessful and,

it was therefore impossible to determine the level of expression. The inability to isolate permanent transfectants is probably indicative of a high level of β -III expression that is lethal. No attempt has been made to isolate permanently transfected clones expressing the β -III tubulin Ser⁴⁴⁴ mutants.

The β -III tubulin modification present in P19 cells transfected with wild-type β -III tubulin, was not specifically identified, but based on its location on the IEF gel, it appears likely the result of Ser⁴⁴⁴ phosphorylation. Only one isoform is detected, and if glutamylation was occurring, several isoforms would be expected. This would suggest that the enzyme(s) responsible for phosphorylation are present in undifferentiated P19 cells and that those for polyglutamylation are developmentally regulated. Little is known about the enzymes responsible for the modifications of β -III tubulin. A casein-kinase II-like enzyme which copurifies with brain Mt proteins is thought to be a likely candidate for β -III tubulin Ser⁴⁴⁴ phosphorylation and casein kinase II has been shown to be present in cycling, undifferentiated cells (Seranno et al., 1987).

Expression of β -III tubulin in which Ser⁴⁴⁴ was substituted with (Glu⁴⁴⁴) to mimic a constitutively-phosphorylated β -III tubulin did not alter subcellular sorting or modulate the incorporation of β -III tubulin into stable Mts in P19 cells. *Pgk-1- β -IIIAla⁴⁴⁴* transfection yielded similar results. Changes in the dynamic properties of these β -III tubulin mutants may exist which are too subtle to be detected by colchicine-

treatment. Direct observations of Mt rates of growth and shrinkage may be required to definitively assess more subtle changes in Mt dynamics (Shulze and Kirschner, 1986; Shulze and Kirschner, 1987).

The results of these transient transfection studies lead to the conclusion that expression of β -III tubulin with a single posttranslational modification is insufficient to confer colchicine-stability to Mts in undifferentiated P19 cells. It is possible that polyglutamylation would result in increased stability. Alternatively, the developmental regulation of Mt dynamics may require the coordinated expression and posttranslational modifications of tubulin and MAPs to provide biochemical forms of each which favour specific interactions, each combination conferring a specific dynamic property.

FUTURE PROSPECTS

Further testing is necessary to assess the role of β -III tubulin modifications in modulating the assembly of the cytoskeleton during neuronal differentiation. The subcellular distribution of β -III tubulin isoforms during neurogenesis has not been examined. Not all β -III tubulin present during neurogenesis is modified in the same way. Two other cytoskeletal proteins, MAP1B and neurofilament-H, are expressed in developing axons and dendrites, but are phosphorylated within axons only (Sato-Yoshitake *et al.*, 1989; reviewed by Cambray-Deakin *et al.*, 1990). In the same way, isoforms of β -III tubulin may exhibit distinct intracellular distribution during

neuronal development.

Two principal strategies could be used to investigate this possibility:

1. Generation of antibodies to the Ser⁴⁴ phosphorylated, Tyr⁴⁶ phosphorylated and Glu⁴⁷ polyglutamylated form of Class III β -tubulin.

These antibodies could be used in immunofluorescence microscopy studies to characterize the developmental appearance and subcellular distribution of β -III tubulin isoform(s) during the differentiation of neuronally-induced P19 cells. Such a study would reveal whether there is sorting of these tubulin isoforms in the developing neuron. The antibodies could also be used for immunoblotting, to identify specific isoelectric variants in soluble and polymeric fractions of P19 neurons.

2. Preparation of epitope "tagged" β -III tubulin cDNA constructs for expression in P19 neurons, one wild-type β -III tubulin, and the others altered by site-directed mutagenesis: Ser⁴⁴, Tyr⁴⁶, and Glu⁴⁷ (See Figure 1), with subsequent comparison of subcellular sorting during neurogenesis.

In addition to the epitope tag, this plan requires the ability to express the gene of interest in P19 neurons. The *Ta1* α -tubulin promoter would serve this purpose (See appendix).

Preliminary attempts to epitope tag β -III tubulin at the amino terminus have been unsuccessful. P19 cells were transfected with the RSV-6myc- β -III tubulin expression vector,

which carries a six-repeat c-myc tag at the 5' end of β -III tubulin (see Figure 5) and double-immunolabelled using the TuJ1 antibody and 9E11, a rabbit monoclonal antibody which recognizes an epitope within the c-myc protein tag. No labelling of β -III tubulin or c-myc was observed. When whole cell extracts of transfected cells were prepared for SDS-PAGE followed by western blotting, no β -III tubulin or c-myc protein was detected. Sequencing of the RSV-6myc- β -III tubulin expression vector confirmed that the c-myc tag was "in-frame". Some difficulty was experienced when sequencing through a guanine/cytosine (GC) rich region of the c-myc tag, and it is not known if this is the result of secondary DNA structure. A variety of changes to the transfection procedure were devised to facilitate expression of β -III tubulin with this expression vector. In one experiment, transfections were performed using various quantities (2 μ g to 30 μ g) of RSV-6myc- β -III tubulin and in another, cells were fixed at various time points after transfection (8 hr, 12 hr, 24 hr, 36 hr and 24 hr). P19 cells growing on coverslips were transfected directly to bypass the trypsinization step. No β -III tubulin or c-myc protein was detected following these alterations in procedure. No further analysis was performed.

The first four amino acids of β -III tubulin were removed to prepare this expression vector, thereby bypassing the autoregulatory mechanism for β -tubulin. It is not known if 6-myc β -III is transcribed in P19 cells, if expression of myc- β -III tubulin is lethal, or if the protein is expressed and rapidly

degraded.

Recently, a strategy has been used to successfully prepare an assembly competent mammalian β -tubulin with an hemagglutinin 'tag' at the carboxyl-terminus (Gonzalez-Garay and Cabral, 1995). To distinguish the expression of endogenous β -III tubulin from the β -III cDNA constructs, the sequence coding for the hemagglutinin antigen (30 base pairs) could be cloned into the Apal restriction site found at β -III tubulin codon 449 to serve as an 'immunological tag'. When expressed, this protein can be detected with an antibody to the hemagglutinin 'tag'.

Using immunofluorescence microscopy, IEF and immunoblotting, neuronally-induced stable transfectants would be examined for the subcellular sorting of/or selection against the mutant β -III into distinct Mt arrays. Colchicine-stability, response to taxol, and cellular distribution during neuronal morphogenesis would be examined.

These experiments will test the importance of modified sites on the sorting of β -III tubulin during neurogenesis by revealing distinctive differences in subcellular distribution or colchicine-stability. The β -III tubulin mutants may exhibit a different sorting pattern than the wild-type β -III, and may, for example, be preferentially incorporated into the Mts of developing axons. Alternatively, the absence of distinctive sorting will indicate that modifications of β -III tubulin are not related to subcellular sorting of β -III during neurogenesis.

APPENDIX

T α 1-TUBULIN PROMOTOR ACTIVITY IS RESTRICTED TO NEURONS IN RA-INDUCED P19 EC CELLS

While P19 cells are readily transfected by calcium phosphate/DNA precipitation, stable expression of transfected genes following neuronal differentiation has been difficult to achieve, regardless of the promoter used (McBurney, 1993). Miller et al. (1994) have demonstrated the neuron-specific activity of the T α 1 α -tubulin promoter in transgenic mice. Of four α - and 5 β -tubulin genes expressed in mammalian brain, Class II and III β -, and T α 1 α -tubulin demonstrate similar regulation during neurogenesis (Jiang and Oblinger, 1992). This suggested that it may be possible to utilize the T α 1 α -tubulin promoter in P19 cells induced to differentiate along a neuronal pathway.

In this immunofluorescence microscopy study, Class III β -tubulin was used as a marker for neuronal differentiation, and the tissue-specific expression of *lacZ(n)* was examined following RA-induction of P19-T α 1-*lacZ(n)* clones. P19-T α 1-*lacZ(n)* neurons express β -galactosidase concomitant with neurite outgrowth. This is the first report of neuron-specific promoter activity in transfected RA-induced P19 cells. The regulated activity of the T α 1 α -tubulin promoter may be exploited to investigate the biological role of cytoskeletal proteins during neurogenesis.

METHODS AND MATERIALS

PLASMIDS

1. *Tα1-lacZ(n)* in pUC19

This expression vector (Miller et al., 1994) was kindly provided by Dr. F. Miller, Montreal Neurological Institute, McGill University, Montreal. The 1.2 kb fragment of the 5' flanking region of the rat *Tα1 α-tubulin* gene was fused to the *lacZ* gene carrying an SV40 nuclear localization signal (Figure 4). *lacZ* was used as a reporter gene to assess promoter activity.

2. *Pgk-1-neo^r* resistance in PUC19

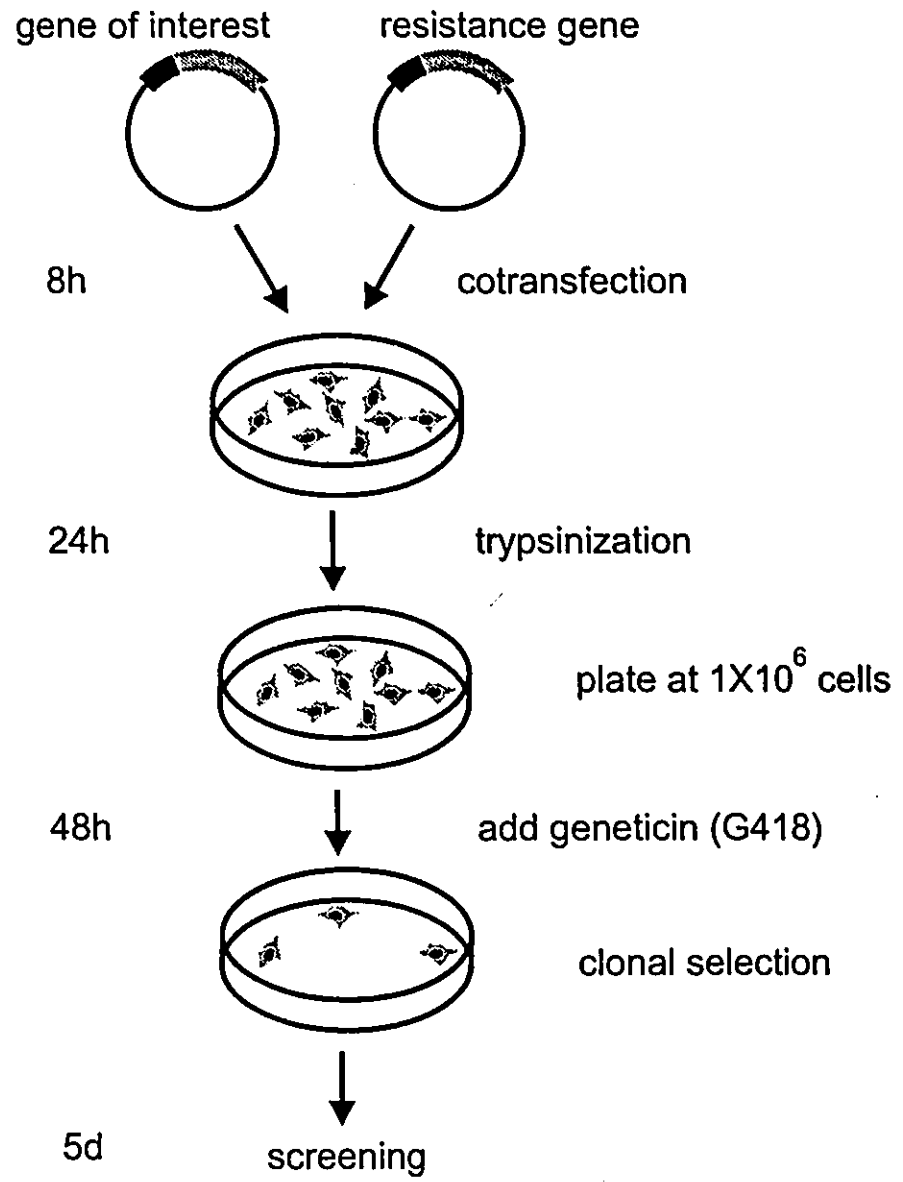
This expression vector was kindly provided by Dr. M. McBurney, University of Ottawa, and consists of the *Pgk-1* promoter ligated to the *neo^r* gene which affords resistance to the neomycin analogue geneticin (G418) and permits selection of stable transfected cells. The *Pgk-1* gene provides a poly(A)-adenylation signal.

ISOLATION OF STABLE TRANSFECTANTS

To isolate stable transfectants, P19 cells were co-transfected with two plasmids: 13 μ g *Tα-1lacZ(n)* and 2 μ g *Pgk-1-neo^r* using the method described previously. Any cell which takes up DNA during transfection will likely take up two separate plasmids, even if these plasmids are unlinked (Wigler et al., 1979).

Neomycin-resistant cells were selected following 5 days of

Figure 23. Summary of selection of clones permanently transfected with gene of interest.



growth in the presence of 450 $\mu\text{g/mL}$ G418, and maintained in the continued presence of 450 $\mu\text{g/mL}$ G418 (Figure 23). As colonies formed, they were selected and cultured in 24-well plates, followed by 60-mm dishes and finally grown to semi-confluency in 100-mm dishes.

Each clone was induced to differentiate along a neuronal path with 10^{-6} M RA (as described previously) and screened for nuclear-lacZ expression by histochemical staining with X-gal as described previously. Clones which expressed β -galactosidase were further characterized by double-immunofluorescence microscopy using an antibody to β -III tubulin (TuJ1) and an antibody to β -gal.

PRIMARY ANTIBODIES

Anti- β -III tubulin (TuJ1) (gift from Dr. A. Frankfurter): a mouse monoclonal (IgG) which recognizes Class III β -tubulin, was diluted 1:300.

Anti- β -galactosidase (Cappel, Organon Teknika, West Chester, PA): rabbit polyclonal (IgG) which recognizes β -galactosidase, diluted 1:500.

SECONDARY ANTIBODIES

1. Goat anti-rabbit (IgG), conjugated to indocarbocyanine (Cy3), cross-adsorbed to mouse (Jackson), used at 1:500.
2. Goat anti-mouse (IgG), conjugated to fluorescein isothiocyanate (FITC), cross-adsorbed to rabbit (Jackson), used at 1:100.

IMMUNOFLUORESCENCE LABELLING

For P19-*Tα1-lacZ(n)* clones immunofluorescence labelling was performed using the method previously described with several changes: 0.2% Triton X-100 was added to all antibody and rinse solutions, and double-labelling was performed sequentially. The anti- β -galactosidase antibody was used first, followed by the appropriate secondary; then TuJ1 followed by the appropriate secondary antibody. All antibody incubation times were 45 min. Hoechst staining was not performed.

CONFOCAL MICROSCOPY

P19 *Tα1-lacZ(n)* clones were examined using an upright Leica CLSM (confocal laser scanning microscope) system, with the pinhole set small enough to obtain approximately 1 μ m sections. Serial optical sections were collected simultaneously for β -III and β -gal labelling through the entire depth of field, to ensure all staining was captured in focus. Optical sections were composited to produce an extended focus image. Extended focus images for β -III and β -gal labelling were combined to produce a 2 colour image showing both β -III and β -gal staining in the same field. Images were recorded in tagged image file format (TIFF) with the resolution of 512 columns X 512 lines X 256 colours.

RESULTS

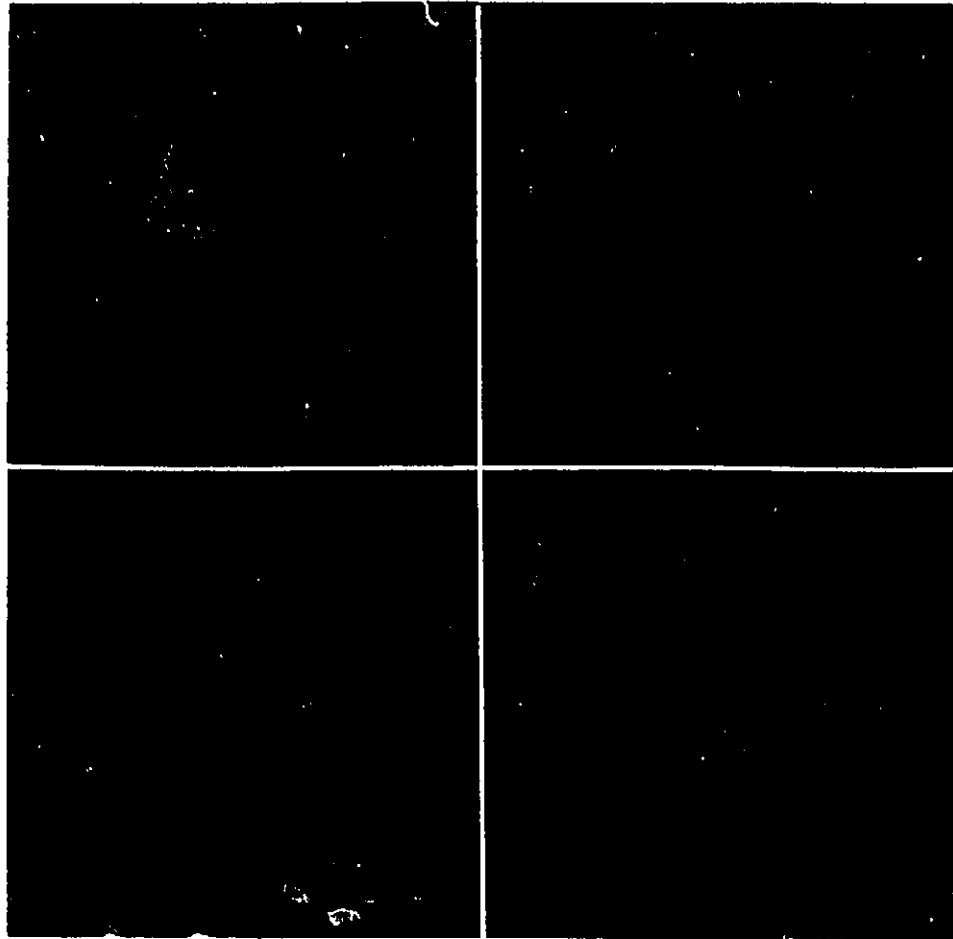
Thirty-six P19-*Tα1-lacZ(n)* clones were selected, differentiated and screened for nuclear-*lacZ* expression by histochemical staining with X-gal (Figure 23). A total of seven

clones exhibited β -galactosidase activity following the addition of RA. Two of these (#19 and #30) were further characterized by double-immunofluorescence microscopy using an antibody to β -III tubulin (TuJ1) and an antibody to β -gal.

Three coverslips from 3 independent culture experiments with clone #30 were examined, and approximately 6 fields on each coverslip were assessed. Cells immunolabelled with anti- β -gal were counted, and then the fluorescence filter was changed and the number of cells labelled with the antibody to neuronal β -III tubulin were counted to determine the percentage of cells exhibiting colocalization.

At day 0, a few cells expressed low levels of β -III tubulin, a few low levels of β -gal and a few expressed both proteins (Figure 24A). Class III β -tubulin was detected following 48 hours of RA, although no obvious staining with anti- β -gal was observed (Data not shown). At day 3, the first appearance of intense nuclear labelling with anti- β -gal was noted in approximately 75% of β -III tubulin-labelled neurons (Figure 24B). Approximately 20% of β -III positive neurons (many with short or absent extensions) showed little β -gal staining (arrows), and approximately 5% of β -gal positive cells showed no β -III tubulin labelling (arrowhead). In day 8 clones (Figure 24C) intense nuclear labelling with anti- β -gal was present in most β -III tubulin-labelled neurons. By day 12, 95% of neurons exhibited nuclear labelling with anti- β -gal (Figure 24D) and less than 5% of β -gal (arrowhead) or β -III tubulin (arrow) positive cells did not show colocalization with both markers.

Figure 24. Confocal microscopy of RA-induced P19-T α 1-lacZ(n) clones double-labelled with antibodies to Class III β -tubulin (green) and β -galactosidase (red). Labelling was visualized through the entire depth of the sample by integrating 1 μ m optical sections. Low level expression of β -III tubulin and β -gal in undifferentiated clones (A). Colocalization of β -gal and β -III tubulin is first evident at day 3 (B). Some neurons are not labelled by anti- β -gal (arrows), and some β -gal positive cells are not labelled by anti- β -III tubulin (arrowhead). By day 8 (C) colocalization of β -gal and β -III tubulin is readily apparent. A β -gal positive nuclei (arrowhead) not labelled by anti- β -III (C). Day 14 (D) colocalization of β -gal and β -III tubulin in almost all cells. Very few β -gal positive nuclei (arrowhead) are not labelled by anti- β -III and few neurons (arrow) are not labelled by anti- β -gal.



CONCLUSION

1. The $T\alpha 1$ tubulin promoter appears to be activated specifically following RA-induction in P19 neurons.
2. The majority of P19- $T\alpha 1$ -lacZ(n) neurons express detectable levels of β -galactosidase concomitant with neurite outgrowth.
3. The results reported here parallel the results in transgenic mice and emphasise the usefulness of the P19 cell model system in investigating the early molecular events of neuronal differentiation.

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