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Mutually exclusive expression of β_{III}-tubulin and vimentin in adrenal cortex carcinoma SW13 cells

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Abstract During embryogenesis, the maturation of neuroblasts into neurones is accompanied by the down-regulation of vimentin and by the expression of neuronal microtubular proteins. Here, we show that human adrenal cortex SW13 cells express β_{III}-tubulin, MAP2b and tau. Analysis of vimentin-positive and -negative subclones of SW13 cells revealed that, under defined cultured conditions, β_{III}-tubulin and MAP2b were present only in vimentin-deficient cells and that β_{III}-tubulin repression occurred at the transcriptional level in vimentin-positive cells. These results suggest that vimentin repression and β_{III}-tubulin expression are co-ordinated by an upstream mechanism relevant to the control of cytoskeletal protein expression during neuronal development.

Key words: β_{III}-Tubulin; Vimentin; Adrenal cortex; Carcinoma; Cytoskeleton; Neurogenesis

1. Introduction

During embryogenesis, differentiation of neuroblasts into neurones is accompanied by the sequential expression of cytoskeletal proteins that are required for morphological transformation, in particular, for the extension of axons and dendrites. Developmental regulation of cytoskeletal proteins is especially clear for intermediate filament proteins. Nestin is the first intermediate filament protein to be expressed in neuroblasts [1] and is gradually replaced by vimentin (for a review, see [2]). Neuronal intermediate filament proteins are then progressively expressed in post-mitotic neurones, first α-internexin, followed by neurofilament subunits. Initially, NF-L and NF-M are co-expressed, the third subunit, NF-H appears shortly before axons reach their targets.

Like intermediate filament proteins, individual tubulin isoforms have a regulated pattern of expression during maturation of neuronal progenitor cells into neurones and this is best illustrated for β_{III}-tubulin. Like other tubulin isoforms, β_{III}-tubulin is coded by its own gene and differs from other β-tubulin isoforms by a specific 15 amino acid C-terminal sequence [3]. β_{III}-Tubulin expression starts at the late stages of neuronal differentiation and is sustained in mature neurones; in addition, β_{III}-tubulin expression begins earlier in peripheral neurones than in central neurones [4,5]. The role of β_{III}-tubulin in the properties and functions of microtubules is still unclear, but it may modulate their dynamic properties. In vitro studies have shown that microtubules assembled from $\alpha\beta_{III}$-tubulin are considerably more dynamic than microtubules made of α-tubulin combined with other β-tubulin isoforms [6]. Therefore, β_{III}-tubulin may be implicated in the regional variations in dynamic properties of microtubules that exist along growing axons [7].

Expression of β_{III}-tubulin during neurogenesis is controlled by a negative regulatory element, the neurone-restrictive silencer element (NRSE), a *cis*-acting silencer thought to mediate the transcriptional repression of multiple neuronal-specific genes [8–10]. On the other hand, expression of the vimentin gene exhibits a complex regulation, involving the combination of both positive and negative elements [11–15]. Here, we show that β_{III}-tubulin is expressed in a cell line originating from an adenocarcinoma of the human adrenal cortex and that transcriptional activation of β_{III}-tubulin is conditional to transcriptional repression of vimentin. These results suggest that vimentin repression and β_{III}-tubulin expression are tightly linked at the transcriptional level. Such a co-ordinated upstream mechanism would be highly significant for the control of sequential cytoskeletal protein expression during neuronal development.

2. Materials and methods

2.1. Cell culture

The human adrenal adenocarcinoma cell line, SW13, was obtained from the European Animal Cell Culture Collection (Porton Down, UK). SW13 cells express vimentin in a mosaic pattern and the original population comprises vimentin-positive and vimentin-negative cells [16]. A vimentin-negative subclone of SW13 cells, SW13vim$^-$, was selected after limiting dilution cloning [17] and a vimentin-positive clone (SW13vim$^+$, cl.1 [18]) was kindly provided by Dr Robert Evans (University of Colorado, Boulder, CO, USA). Cells were grown in DMEM supplemented with 10% heat-inactivated foetal bovine serum (Gibco BRL/Life Technologies) in a humidified atmosphere of 5% CO_2/95% air. In some experiments, cells were cultured in phenol red-free DMEM supplemented with dextran-coated charcoal (DCC)-treated serum.

2.2. RNA isolation and Northern blotting

Total RNA was extracted from cultured cells using the phenol/chloroform/isoamyl alcohol method [19]. For Northern blotting analysis, 15 μg of total RNA from each sample was separated on 1% agarose/formaldehyde gels and vacuum-blotted onto GeneScreen-Plus Nylon membranes (DuPont NEN). Membranes were then hybridised with a ^{32}P-labeled vimentin probe. The vimentin probe used was a 1.8 kb human vimentin cDNA fragment corresponding to the entire coding region and was excised by *Eco*RI restriction digestion from the pFB4.2 plasmid [20] (obtained from Dr Robert Evans). Labeling of the probe was carried out using the Prime-It™ II Random Primer Labeling kit (Stratagene) according to the manufacturer's instructions. After hybridisation, membranes were washed to a final stringency of

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0.2×SSC. For autoradiography, membranes were exposed for 1 day on Kodak X-Omat film with an intensifying screen at −70°C.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of tubulin transcripts

For RT-PCR, total RNA was treated with DNase I (amplification grade, Gibco BRL) and reverse-transcribed with 15 U of AMV reverse transcriptase (Promega) for 45 min at 42°C in 10 mM Tris–HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 1 mM of all four dNTPs containing 25 U of rRNasin. PCR was carried out using 3 μl of the reverse transcription reaction mixture in a final volume of 50 μl using 2.5 U of cloned *Pyrococcus furiosus* (*Pfu*) DNA polymerase (Stratagene). PCR amplification mixture contained 20 pmol of each primer (see below), 200 nM of each dNTP, 20 mM Tris–HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 and 0.1 mg/ml BSA. Double-stranded DNA was denatured at 95°C for 5 min and 30 PCR cycles were performed with the following program: 94°C for 30 s, 65°C for 30 s and 72°C for 2 min. Finally, reaction mixtures were incubated at 72°C for 3 min. PCR products were analysed by electrophoresis in 2.5% low melting point NuSieve agarose gels (FMC BioProducts, Rockland, ME, USA). ϕX174 RF DNA/*Hae*III fragments ranging from 72 to 1353 bp were used as DNA size markers.

β_{III}-Tubulin cDNA was amplified with the primers 5′-GCCATCTTCCCGCGCAACCG-3′ (forward) and 5′-CTTGGGGCCCTGGGACTCCG-3′ (reverse). The latter corresponds to the region encoding the isoform-determining 15 C-terminal amino acids of β-tubulin [3] and was derived from the nucleotide sequence of human β_{III}-tubulin (D.W. Cleveland, personal communication). As a control, α-tubulin was also amplified, the primers used were -5′-GCGTGAGTGCATCTCATCCT-3′ (forward) and -5′-GCTGGCGGTAGGTGCCAGTG-3′ (reverse). The α-tubulin primers correspond to a conserved region of human α-tubulin [21] with no homology with β-tubulin. The expected sizes of the resulting amplification products are 195 bp and 253 bp, for β_{III}- and α-tubulin, respectively.

2.4. Western blotting

Total cell extracts were prepared by homogenising the cells in electrophoresis sample buffer (62.5 mM Tris–HCl, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol) at 100°C. For preparation of heat-stable fractions for tau analysis, cells were resuspended in Mes/NaCl buffer (100 mM 2-(*N*-morpholino) propanesulfonic acid, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 M NaCl, 2 mM DTT, 0.1 mM $NaVO_4$, 1 mM PMSF, pH 6.5) and heated at 100°C for 5 min. After centrifugation at $16\,000\times g_{av}$ for 25 min, the pellet was discarded and the supernatant was diluted in electrophoresis sample buffer. Proteins were separated in 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in 10% skimmed milk in phosphate-buffered saline (PBS) for 1 h at 37°C. Subsequent antibody incubations were carried out in PBS containing 5% skimmed milk and 0.5% Tween-20. Membranes were routinely incubated with primary antibodies overnight at 4°C, followed by incubation with the appropriate species-specific secondary antibody conjugated to horseradish peroxidase (Amersham, 1:2000 dilution) for 1 h at room temperature. Membranes were washed three times for 10 min in PBS containing 0.5% Tween-20 between antibody steps. Immunoreactivity was detected using the enhanced chemiluminescence development system (Amersham). The monoclonal antibodies, RPN1102 to vimentin and SDL3D10, specific for β_{III}-tubulin [22] were purchased from Amersham and Sigma, respectively. MAP2b and tau were detected with the rabbit polyclonal antibodies, B9 [23] and TP70 [24], respectively.

3. Results

3.1. Vimentin expression in SW13vim$^+$ and SW13vim$^-$ cells

SW13 cells are derived from a human carcinoma of the adrenal cortex and grow in culture with the characteristic morphology and growth pattern of epithelial cells. Vimentin is the only detectable cytoplasmic intermediate filament protein in SW13 cells and is expressed in a mosaic pattern [16,18]. Subclones of SW13 cells, either expressing vimentin or not, have been selected by limiting dilution from the parent cell line on the basis of the presence or absence of a vimentin filament network detectable by immunofluorescence microscopy [17,18]. The vimentin protein in the two clones used in this study, SW13vim$^+$ and SW13vim$^-$, was analysed by Western blotting of total cell lysates using a monoclonal antibody to vimentin (Fig. 1A). Vimentin was visualised as a single band at approximately 55 kDa in lysates from SW13vim$^+$ cells (Fig. 1A, lane 1). By contrast, under similar conditions, no signal was detected in SW13vim$^-$ cells (Fig. 1A, lane 2). The present study originated as part of an investigation of the effects of steroid hormones on cytoskeletal protein expression, therefore, in some experiments, cells were grown in steroid-depleted medium for 48 h prior to harvesting. This was achieved by using phenol red-free medium supplemented with DCC-treated foetal bovine serum (DCC medium). The pattern of vimentin expression was not affected whether the cells were grown in normal culture medium or in DCC medium (not shown).

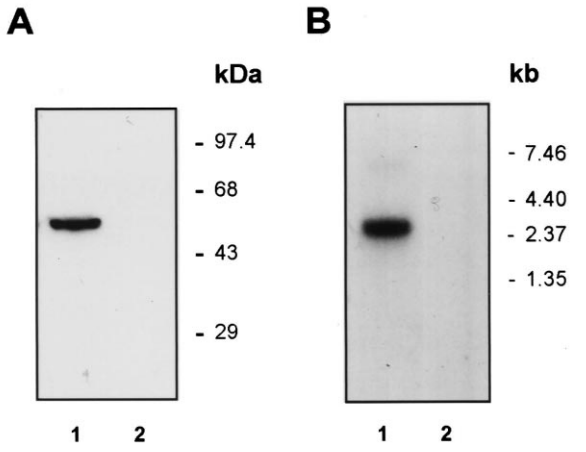

Fig. 1. Transcriptional control of vimentin expression in SW13vim$^+$ and SW13vim$^-$ cells. (A) Western blot analysis of total cell lysates from SW13vim$^+$ and SW13vim$^-$ cells using a monoclonal anti-vimentin antibody confirming the absence of vimentin in SW13vim$^-$ cells. (B) Northern blot analysis of total RNA from SW13vim$^+$ (lane 1) and SW13vim$^-$ (lane 2) cells using a 1.8 kb cDNA probe to human vimentin demonstrating transcriptional repression of vimentin in SW13vim$^-$ cells.

Presence or absence of vimentin in SW13 cells has previously been reported to be determined at the transcriptional level [20]. This was confirmed by Northern blotting for the SW13vim$^+$ and SW13vim$^-$ subclones. Total RNA from SW13vim$^+$ and SW13vim$^-$ cells was analysed using a radiolabeled 1.8 kb cDNA probe corresponding to the entire coding region of the human vimentin gene (Fig. 1B). A single band of approximately 2.5 kb corresponding to vimentin mRNA was detected in RNA from SW13vim$^+$ cells (Fig. 1B, lane 1). In contrast, no hybridisation signal was visible in the sample from SW13vim$^-$ cells (Fig. 1B, lane 2). Taken together, these results confirm that absence of vimentin in SW13vim$^-$ cells results from transcriptional repression and that interconversion, which can occur between vimentin-positive and vimentin-negative cells, was minimal.

3.2. Expression of neuronal microtubular proteins in SW13 cells

An unusual, but apparently common, feature of carcinomas of the adrenal cortex is the expression of neuronal proteins,

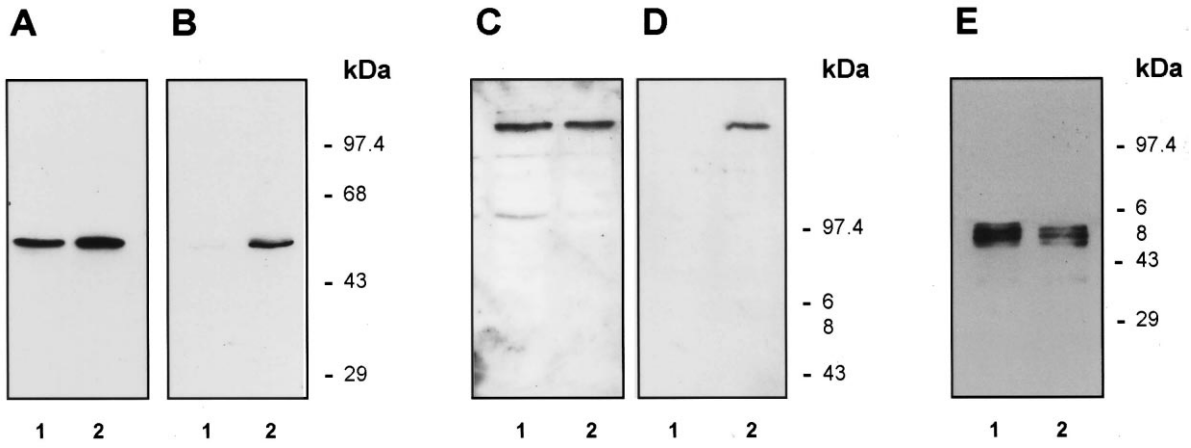

Fig. 2. Expression of neuronal microtubular proteins in SW13vim$^+$ and SW13vim$^-$ cells. (A–D) Total cell lysates and (E) heat-stable preparations from SW13vim$^+$ (lane 1) and SW13vim$^-$ (lane 2) cells. (A, C, E) Cells grown in normal medium; (B, D) cells grown in phenol red-free medium supplemented with DCC-treated serum. Cell lysates were analysed by Western blotting using: (A, B) the β_{III}-tubulin-specific antibody, SDL3D10; (C, D) the B9 antibody to MAP2b; (E) the TP70 antibody to tau.

such as synaptophysin, neurone-specific enolase or NCAM [25]. As SW13 cells are derived from an adrenal cortex carcinoma and as the two subclones, SW13vim$^+$ and SW13vim$^-$, exhibit a differential profile of cytoskeletal protein expression, we sought to determine whether they expressed microtubular proteins normally selectively expressed in neurones. We chose to study the expression of the class III β-tubulin isoform, β_{III}-tubulin, and of two microtubule-associated proteins, the high molecular weight MAP2b and tau. Western blot analysis using an antibody specific for β_{III}-tubulin demonstrated a single band at approximately 55 kDa in SW13vim$^+$ and SW13vim$^-$ cells grown in normal culture medium (Fig. 2A). The amount of β_{III}-tubulin present in SW13vim$^+$ cells appeared to be slightly lower than in SW13vim$^-$ cells. By contrast, when SW13 cells were grown in DCC medium, β_{III}-tubulin was still clearly apparent in SW13vim$^-$ cells (Fig. 2B, lane 2) but was not readily detectable in SW13vim$^+$ cells (Fig. 2B, lane 1).

A polyclonal antibody to MAP2b revealed a single band at 280 kDa on Western blots of extracts from both SW13vim$^+$ and SW13vim$^-$ cells cultured in normal medium (Fig. 2C). The amount of MAP2b present in each sample appeared to be approximately equal in both cell types. When the cells were grown in DCC medium, MAP2b was only detectable in SW13vim$^-$ cells and was not apparent in SW13vim$^+$ cells (Fig. 2D), and thus appears to follow the same pattern of expression as β_{III}-tubulin in SW13 cells. Tau was also found to be expressed in SW13vim$^+$ and SW13vim$^-$ cells and was detected as a set of three bands migrating at 45–55 kDa (Fig. 2E). Unlike β_{III}-tubulin and MAP2b, the pattern of tau expression did not appear to be affected by the type of culture medium used (not shown). Therefore, SW13 cells express a number of microtubular proteins normally restricted to neuronal cells. Moreover, the presence of β_{III}-tubulin and MAP2b in SW13 cells is correlated with the absence of vimentin expression in defined culture conditions.

3.3. Analysis of β_{III}-tubulin transcripts

The absence of detectable β_{III}-tubulin protein in SW13vim$^+$

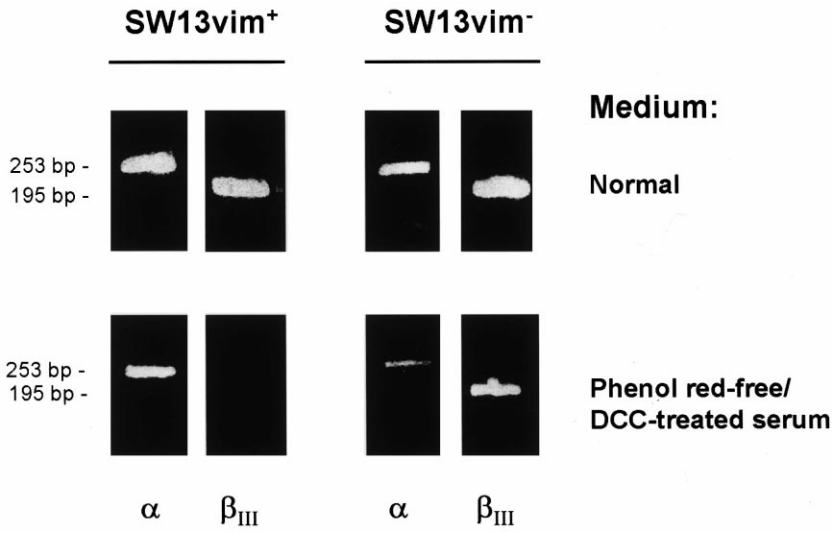

Fig. 3. Transcriptional repression of β_{III}-tubulin in SW13vim$^+$ cells. RT-PCR analysis was performed on total RNA isolated from SW13vim$^+$ and SW13vim$^-$ cells grown in normal medium or in phenol red-free medium supplemented with DCC-treated serum. Tubulin sequences were amplified using pairs of primers specific for α-tubulin or β_{III}-tubulin, yielding fragments of 253 bp and 195 bp, respectively. The sequence of forward and reverse primers is indicated in Section 2.

cells cultured in DCC medium may be the result of protein degradation or of repression at the transcriptional level. To discriminate between these two possibilities, β_{III}-tubulin transcripts were compared by RT-PCR in SW13vim$^+$ and SW13vim$^-$ cells. Amplification of a β_{III}-tubulin-specific sequence was achieved by using a reverse primer corresponding to the sequence encoding the isoform-determining 15 C-terminal amino acids of β_{III}-tubulin. As a control, a cDNA fragment for α-tubulin was also amplified using primers derived from a conserved region of the protein with no homology with β-tubulin. RT-PCR on RNA from cells grown in normal medium produced a fragment of 195 bp corresponding to β_{III}-tubulin for both SW13vim$^+$ and SW13vim$^-$ cells (Fig. 3). In comparison, RT-PCR analysis carried out on RNA from cells grown in DCC medium revealed a PCR amplification product for β_{III}-tubulin in the sample from SW13vim$^-$ cells only; no similar PCR product was observed in RNA from SW13vim$^+$ cells (Fig. 3). Thus, RT-PCR results are consistent with Western blot findings and show that the absence of β_{III}-tubulin in vimentin-positive SW13 cells is the result of repression at the transcriptional level.

4. Discussion

A number of microtubular proteins predominantly expressed in neurones, namely β_{III}-tubulin and the microtubule-associated proteins, MAP2b and tau, were found to be present in SW13 cells. Although mainly neuronal, these proteins are not restricted to neurones. For instance, tau and an isoform of MAP2 are present, albeit at low levels, in astrocytes [26,27]. Outside the nervous system, β_{III}-tubulin is expressed in testes and also in a subpopulation of chromaffin cells of the adrenal medulla [28]. The latter may be explained by the common embryonic origin of chromaffin cells and sensory neurones. Although not arising from the same lineage as cells from the medulla, cells from the zona glomerulosa of the adrenal cortex display some signs of neuroendocrine differentiation, as shown by expression of synaptophysin, neurone-specific enolase or NCAM [25]. Moreover, expression of neuronal proteins appears to be a common feature of carcinomas of the adrenal cortex [25]. Therefore, expression of neuronal microtubular proteins in SW13 cells, that have been established from a carcinoma of the adrenal cortex, is consistent with these observations.

Interestingly, analysis of the two SW13 cell subclones, SW13vim$^+$ and SW13vim$^-$, revealed that β_{III}-tubulin expression was limited to vimentin-deficient cells, when the cells were grown in phenol red-free medium supplemented with DCC-treated foetal bovine serum. Vimentin mRNA was not detected in SW13vim$^-$ cells, indicating that vimentin was repressed at the transcriptional level in this subclone, confirming previous results obtained in a similar vimentin-negative SW13 cell subclone [20]. Likewise, RT-PCR analysis showed that the absence of β_{III}-tubulin in SW13vim$^+$ cells was the result of repression at the transcriptional level. Expression of β_{III}-tubulin at the late stages of neuronal differentiation results from the loss of a functional neurone-restrictive silencer factor (NRSF), the zinc finger *trans*-acting factor binding to the NRSE in the promoter region of the β_{III}-tubulin gene [9,10]. On the other hand, the vimentin promoter region contains several *cis*-acting silencer and anti-silencer regulatory elements [11–15]. Anti-silencing activity overrides the effect of silencers [13,15] and contributes to vimentin induction by mitogenic factors contained in serum that occurs in a number of cell lines [11,14]. For example, mouse neuroblastoma NB2a cells express vimentin in culture [29] and we have found by RT-PCR that they expressed β_{III}-tubulin in DCC medium (not shown). Thus the anti-silencing activity inducing vimentin expression does not result in β_{III}-tubulin repression. More likely, vimentin and NRSF would be under the control of a similar silencer element and SW13vim$^-$ cells would differ from SW13vim$^+$ cells by the presence of a functional silencer-binding protein. This would be consistent with the pattern of vimentin expression in non-metastatic versus metastatic human breast cancer cell lines that appears to be determined by the presence or absence of negative regulation [30]. A common upstream silencing mechanism controlling the expression of NRSF as well as vimentin could therefore explain the mutually exclusive expression of β_{III}-tubulin and vimentin in SW13 cells.

The difference in expression of β_{III}-tubulin in SW13vim$^+$ and SW13vim$^-$ cells was abolished in cells grown in culture medium supplemented with normal, untreated serum. Under these conditions, SW13vim$^+$ cells as well as SW13vim$^-$ cells were found to express β_{III}-tubulin. This suggests that a factor, present in serum and removed by DCC treatment, would activate a transcription factor overriding the effect of a silencing element. The nature of such a factor is unknown at present, but a steroid hormone would be a possible candidate as steroids have been shown to regulate the expression of cytoskeletal proteins in specific subpopulations of neurones. For instance, estrogens up-regulate MAP2 in hypothalamic neurones in culture [31] and testosterone up-regulates an isoform of β-tubulin, β_{II}-tubulin, during axonal regeneration of facial motor neurones after axotomy [32]. However, testosterone or glucocorticoids do not appear to induce β_{III}-tubulin expression in SW13vim$^+$ cells (data not shown). MAP2b seems to follow the same pattern of expression as β_{III}-tubulin in SW13 cells but it is unclear at present whether the absence of the MAP2b protein in SW13vim$^+$ cells is due to transcriptional repression, as it is for β_{III}-tubulin. In contrast to MAP2b, we did not observe any differential regulation of tau expression between vimentin-expressing and non-expressing SW13 cells.

The expression pattern of β_{III}-tubulin and vimentin in SW13 cells appears to recapitulate the program of expression of these two cytoskeletal proteins during neuronal development. Vimentin is only expressed in neuronal precursors and β_{III}-tubulin is characteristic of mature neurones. However, during development, the two proteins overlap during a brief period in newly committed neuroblasts, the stage at which β_{III}-tubulin is first expressed [5,33], and when vimentin is still present [34]. Based on the results obtained in SW13 cells, it can be hypothesised that, in committed neuroblasts, β_{III}-tubulin expression is controlled through the action of an extracellular signal. Later in development, regulation at the transcriptional level through a co-ordinated mechanism, possibly involving silencer elements, would lead to vimentin repression and permanent expression of β_{III}-tubulin in mature neurones.

Since SW13vim$^-$ cells are devoid of cytoplasmic intermediate filaments, they are increasingly used to analyse the assembly properties of neuronal intermediate filaments [35–37]. The presence in SW13 cells of cytoskeletal proteins mainly restricted to neurones should be taken into account in interpret-

ing results obtained with this cell line as this may influence the assembly of neuronal intermediate filaments.

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