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The Expression and Posttranslational Modification of a Neuron-Specific β-Tubulin Isotype During Chick Embryogenesis

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Five β-tubulin isotypes are expressed differentially during chicken brain development. One of these isotypes is encoded by the gene $c\beta_4$ and has been assigned to an isotypic family designated as Class III (βIII). In the nervous system of higher vertebrates, βIII is synthesized exclusively by neurons. A βIII-specific monoclonal antibody was used to determine when during chick embryogenesis $c\beta_4$ is expressed, the cellular localization of βIII, and the number of charge variants (isoforms) into which βIII can be resolved by isoelectric focusing. On Western blots, βIII is first detectable at stages 12–13. Thereafter, the relative abundance of βIII in brain increases steadily, apparently in conjunction with the rate of neural differentiation. The isotype was not detectable in non-neural tissue extracts from older embryos (days 10–14) and hatchlings. Western blots of protein separated by two-dimensional gel electrophoresis (2D-PAGE) reveal that the number of βIII isoforms increases from one to three during neural development. This evidence indicates that βIII is a substrate for developmentally regulated, multiple-site posttranslational modification. Immunocytochemical studies reveal that while $c\beta_4$ expression is restricted predominantly to the nervous system, it is transiently expressed in some embryonic structures. More importantly, in the nervous system, immunoreactive cells were located primarily in the non-proliferative marginal zone of the neural epithelia. Regions containing primarily mitotic neuroblasts were virtually unstained. This localization pattern indicates that $c\beta_4$ expression occurs either during or immediately following terminal mitosis, and suggests that βIII may have a unique role during early neuronal differentiation and neurite outgrowth.

Key words: tubulin heterogeneity, neural differentiation, neuronal microtubules

INTRODUCTION

Microtubules are involved in a diverse variety of processes, including the segregation of chromosomes during mitosis and meiosis, flagella and ciliary motility, organelle transport, and the maintenance of cell shape [Dustin, 1984]. The organization of these polymers into distinct arrays, in different intracellular regions, and at different times during the cell cycle indicates that their assembly and disassembly is under strict temporal and spatial control. Because there is a continuous flux between microtubules and their subunit pools, shifts in the monomer to polymer equilibrium ratio can result in extensive and rapid cytoskeletal reorganization [Mitchison and Kirschner, 1988]. The variables involved in modu-

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lating the timing, rate, and magnitude of such shifts in vivo are still not completely identified.

The principal structural subunit from which microtubules are assembled is the tubulin heterodimer, consisting of similar 50 kDa globular subunits designated α- and β-tubulin. In most eukaryotes, but particularly higher vertebrates, both α-tubulin and β-tubulin exhibit considerable polymorphism. Both subunits are encoded by small multigene families, and both are subject to posttranslational modification, including the addition and subsequent removal of the C-terminal tyrosine on α-tubulin [Barra et al., 1974; Raybin and Flavin, 1975], the acetylation of α-tubulin [L'Hernault and Rosenbaum, 1985; Piperno and Fuller, 1985; Black and Keyser, 1986], the glutamylation of α-tubulin [Eddé et al., 1990], and the phosphorylation of β-tubulin [Eipper, 1974; Gard and Kirschner, 1985; Eddé et al., 1989; Ludueña et al., 1988]. Additionally, the expression of different tubulin genes is differentially regulated during development and is in part either tissue or cell specific [Bond et al., 1984; Havercroft and Cleveland, 1984; Lewis et al., 1985; Lewis and Cowan, 1988; Sullivan, 1988]. Whether the different α- and β-tubulin isotypes are functionally equivalent and interchangeable or whether isotypic differences in primary sequence signify unique functional properties is currently unclear [Cleveland, 1987; Sullivan, 1988].

The differential expression of multiple tubulin genes and the extent of posttranslational modification is reflected in the number and relative abundance of different charge variants (isoforms) which can be resolved by high resolution isoelectric focusing. Vertebrate brain tubulin exhibits the most extensive isoform heterogeneity [Gozes and Littauer, 1978; Gozes and Sweadner, 1981; Denoulet et al., 1982; Wolff et al., 1982; Field et al., 1984]. Specifically, adult mammalian brain tubulin has been resolved into more than 20 isoforms, of which the number of differently charged β-tubulin species is roughly double that of differently charged α-tubulin species [Field et al., 1984; Wolff et al., 1982]. In the case of β-tubulin, the number of isoforms is more than twice the number of expressed genes. Since there is no evidence to indicate that these isoforms result even in part from the alternative splicing of a single gene, more than half of the β-tubulin isoforms are generated by posttranslational modification. These studies have also demonstrated that with neural development the composition of the β-tubulin pool changes substantially with respect to the number, pI, and relative abundance of β-tubulin isoforms. Whereas embryonic brain tubulin can be resolved into three β-tubulin isoforms, adult brain tubulin can be resolved into 14 [Denoulet et al., 1982; Wolff et al., 1982]. Clearly, this developmentally dependent increase in β-tubulin charge heterogeneity signifies extensive and regulated posttranslational modification. Neither the isotypic identity of these different β-tubulin isoforms nor the modifications involved have been conclusively established.

We are interested in determining 1) the extent to which each β-tubulin isotype contributes to the overall pattern of charge heterogeneity, 2) the specific posttranslational modifications involved, 3) when these modifications occur, and 4) the locations of these modifications. To begin to answer these questions, we have chosen to study the β-tubulin isotype designated as βIII [Sullivan and Cleveland, 1986; Sullivan, 1988]. This isotype is the only β-tubulin expressed in avian and mammalian brain whose expression is neuron-specific [Sullivan et al., 1986; Burgoyne et al., 1988; Sullivan, 1988]. Further, βIII differs by approximately 10% in amino acid sequence from the other four β-tubulin isotypes present in brain [Sullivan, 1988], and is selectively phosphorylated on a C-terminal serine residue during neurite outgrowth by neuroblastoma cells and primary cultured neurons [Gard and Kirschner, 1985; Ludueña et al., 1988; Eddé et al., 1989]. In several recent studies we have localized this protein by immunohistochemistry in the developing chick trigeminal system [Moody et al., 1989] and in a number of primary tumours and tumour cell lines [Herman et al., 1989; Katsetos et al., 1989]. These studies indicate that βIII is immunologically detectable immediately after neuroblasts have completed their final mitotic cycle. In this report, we establish definitively the specificity of the anti-βIII antibody (TuJ1) and provide an extensive description of the localization pattern of the isotype in early stage chick embryos. Additionally, we have determined the onset of expression and relative abundance of βIII during chick brain development and show that with development this protein becomes electrophoretically heterogeneous. In conjunction with our earlier studies, we conclude that βIII is synthesized in postmitotic neuroblasts, that its presence is coincident with terminal mitosis and the initial stages of neurite outgrowth, and that it is a substrate for developmentally regulated, multiple-site posttranslational modification.

MATERIALS AND METHODS

Monoclonal Antibodies

Three anti-β-tubulin mouse monoclonal antibodies were used in this study, DM1B(IgG1), Tu27B(IgG1), and TuJ1(IgG2a). DM1B was obtained from Amersham (Arlington Heights, IL). The TuJ1 secreting clone was isolated from hybrids produced by the fusion of SP2/0 myeloma cells with splenocytes of Balb/c mice immunized with taxol-assembled microtubules. The immunuzation, fusion, and screening procedures were per-

formed essentially as described previously for Tu27B [Caceres et al., 1984].

Ascites fluid from Pristane-primed female Balb/c mice was clarified by centrifugation at 40,000*g* for 30 min at 25°C. A relatively pure IgG fraction was obtained first by a modification of the caprylic acid (n-octanoic acid) precipitation procedure described by McKinney and Parkinson [1987]. Briefly, the supernatant was diluted with 4 volumes of 60 mM NaOAc, pH 4.0, and the pH further adjusted to 4.5 with NaOH. Caprylic acid (25 μl/ml) was added slowly while stirring. The solution was stirred for an additional 30 min and then insoluble material was removed by centrifugation (40,000*g*, 30 min, 4°C). The supernatant was diluted 1:9 with 10× PBS, pH 7.4, and the pH adjusted to 7.4. This solution was fractionated at 4°C with $(NH_4)_2SO_4$ (45% saturation). The precipitate was collected by centrifugation (40,000*g*, 30 min, 4°C), resuspended in a minimum volume of PBS containing 0.1 M PMSF and 1 mM EDTA, and dialyzed overnight with 2 changes. The dialysate was further dialyzed against 100 volumes of 1.5 M glycine, 3 M NaCl, pH 8.9, for 1–2 h before separation on a recombinant protein A-Sepharose column (Repligen, Cambridge, MA) equilibrated in the same buffer. IgG subclasses were sequentially eluted from the column with 100 mM citric acid at pH 6.0, 5.0, 4.0, and 3.0. TuJ1 (IgG2a) and Tu27b (IgG1) elute at pH 5.0 and 4.0, respectively. IgG concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of $E\ \frac{1\%}{1cm} = 14$. Pooled fractions were dialyzed exhaustively against 50% glycerol in PBS, adjusted to a concentration of 1 mg/ml, aliquoted and stored at −80°C.

Protein Preparation

Microtubule protein was obtained from adult rat brain by two cycles of temperature-dependent assembly and disassembly [Shelanski et al., 1973]. Tubulin was purified from twice-cycled microtubules by phosphocellulose chromatography [Weingarten et al., 1975]. Taxol-assembled microtubules (TMT) were prepared from the supernatants of 14 day chick embryo forebrain homogenates [Vallee, 1982]. Briefly, the forebrains were homogenized in 100 mM Pipes-NaOH, pH 6.9, containing 1 mM $MgSO_4$, 2 mM EDTA, 1 mM DTT, 0.1 mM GTP, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin (HB) at 4°C with a Tekmar SDT Tissuemizer. The homogenate was clarified by centrifugation (100,000*g*, 45 min, 4°C) and the supernatant adjusted to 10 μM taxol and 1 mM GTP. Tubulin was polymerized at 37°C for 30 min. The taxol-assembled microtubules were pelleted by centrifugation (100,000*g*, 30 min, 25°C). To obtain cytosolic extracts, organs were dissected from decapitated chick embryos (14 days incubation), weighed, and homogenized (1 g/ml) in HB. The supernatant was collected and reduced in an equal volume of 2× Laemmli reducing solution (100°C, 5 min) [Laemmli, 1970]. For whole SDS-extracts, chick embryos were staged according to the criteria of Hamburger and Hamilton and either the heads (stages 12, 16, 18, 23) or the brains (day 14) were homogenized in 1× Laemmli reducing solution (100°C). The homogenates were clarified by centrifugation (100,000*g*, 45 min, 25°C).

Phosphocellulose purifed rat brain tubulin was digested with subtilisin (Sigma, St. Louis, MO) exactly as described by Sackett et al. [1985] at an enzyme to tubulin ratio of 1:100 (wt/wt).

Crosslinking of tubulin with N, N′ethylene bis (iodoacetamide) (EB1) was performed as described by Ludueña et al. [1982].

Production of Cloned Fusion Proteins Containing β-Tubulin Isotype-Defining Sequences

The construction of plasmids used for producing fusion proteins containing β-tubulin isotype-defining sequences and the production of these proteins is described in Lopata and Cleveland [1987]. Each fusion protein contained the N-terminal 32 kDa of the bacterial protein *trp E* linked to the β-tubulin sequence beginning at residue 345 and continuing through to the normal C-terminus. Bacterial extracts containing the fusion proteins were separated by SDS-PAGE, Western blotted, and probed with TuJ1. Binding of the primary antibody was visualized by autoradiography after incubation of blots with ^{125}I-protein A.

Gel Electrophoresis and Immunoblotting

Discontinuous SDS-PAGE was performed according to Laemmli [1970] with 7.5% separating gels and 3% stacking gels. After separation, proteins were transferred electrophoretically to Immobilon-PVDF membranes (Millipore, Bedfore, MA) as described by Dunn [1986]. Membranes were stained either with Amido Black (0.1% Amido Black, 45% methanol, 7% acetic acid) or colloidal gold (Diversified Biotech, Newton Centre, MA). For immunological detection, membranes were incubated first in 5% non-fat dry milk in PBS, pH 7.4 (30 min), and then exposed to the primary antibody overnight (4°C). Thereafter, the membranes were washed thoroughly with PBS and exposed to peroxidase-conjugated goat anti-mouse IgG (γ-chain specific) (Kirkegaard and Perry, Gaithersburg, MD) in 5% non-fat dry milk in PBS (3 h). Then, the blots were washed in PBS and placed in a substrate solution containing 0.0075% (v/v) H_2O_2, 0.04% (w/v) 3,3′ diaminobenzidine tetrahydrochloride dihydrate (Aldrich, Milwaukee, WI) in 50 mM Tris-HCl and 10 mM imidazole, pH 7.6. The reaction was stopped after color development by immersing the membrane in cold distilled water.

High-resolution isoelectric focusing was performed using slab gels (125 × 0.5 mm) containing 8.5 M urea, 5% acrylamide (5% T, 3% C), 2.5% ampholytes (Pharmalyte, pH 4.5–5.4, Servalyte, pH 5–5.5, LKB, pH 5–7, at 5:3:2), 1.5% Nonidet P-40 (NP-40). The gels were cast on a polyester support backing (Gel Bond, FMC, Rockland, ME), overlayed with H_2O-saturated 2-butanol and allowed to polymerize for at least 4 h before use. Gels were prefocused using an LKB Ultraphore horizontal IEF apparatus with an LKB Macrodrive 5 constant power supply for 45 min (10W constant power, 17°C). The cathodlyte was 0.1 M NaOH, the anodlyte was 0.04 M (DL)-glutamic acid, and the interelectrode distance was 110 mm. Fourteen day embryonic chick brain TMT were dissolved directly in IEF sample buffer (9.16 M urea, 5% β-mercaptoethanol. 2.5% ampholytes, 2% NP-40). Proteins from whole SDS-extracts of stage 18, 23, and day 14 embryos were precipitated by adjusting the extracts to 80% acetone (−20°C). The precipitated proteins were pelleted by centrifugation (30,000*g*, 30 min, 4°C) and dissolved in IEF sample buffer. Samples were loaded 20 mm from the cathode into silicone sample wells (Serva, Westburg, NY). The gels were focused for 9 h with the same parameters used for prefocusing. Thereafter, gels were fixed (10% trichloroacetic acid, 5% 5-sulfosalicyclic acid) for 45 min, washed (40% methanol, 10% acetic acid) for 1 h, and stained with colloidal Commassie blue G250 stain [Neuhoff et al., 1988]. The pH gradient of each gel was estimated by cofocusing Pharmacia carbamylated IEF standards (carbonic anhydrase, pH range 4.8–6.7). The pH range was pH 4.9–6.1. For two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), 2–3 mm strips were cut from the stained IEF gels, equilibrated in 1 × Laemmli reducing solution, placed on top of a stacking gel, and sealed with 1% agarose containing 1 × Laemmli reducing solution. Following separation, proteins were transferred to Immobilon PVDF membranes and stained by one of the procedures described above.

Densitometry

To determine the relative abundance of βIII during embryonic neural development, SDS extracts of either the heads or brains of chick embryos at stages 12, 14, 16, 18, and 23, and day 14 were equated first for the amount of total tubulin. Sample loadings were selected by scanning DM1B-stained immunoblots containing serial dilutions of each extract with an LKB GSXL laser densitometer. Volumes yielding approximately equivalent intensity values within the linear range were chosen for futher use. Then, companion immunoblots containing normalized extracts stained with either TuJ1 or DM1B were scanned, and a ratio of the intensity values for each stage was computed.

Immunocytochemistry

Chick embryos were removed from eggs and staged according to the criteria established by Hamburger and Hamilton [1951] under a dissecting microscope. Embryos at stages 17–26 were fixed by immersion for 24 h at 4°C in a fixative containing 3% paraformaldehyde, 3% sucrose in phosphate-buffered saline (PBS). Following fixation, the embryos were washed in PBS and immersed in 30% sucrose in PBS at 4°C until further processing. The embryos were sectioned in a cryostat at a thickness of 12–16 μM and mounted on gelatin-coated glass slides.

The sections were washed in PBS for 24 h at 4°C and then permeabilized for 15 min in 0.4% Triton X-100. The permeabilized sections were incubated in 5% bovine serum albumin (BSA) for 30 min, washed 3× in PBS, and then incubated with the primary antibodies (TuJ1 or Tu27b) in PBS containing 0.1% BSA for 24 h at 4°C or 3 h at room temperature. After incubation in primary antibody, the sections were washed in PBS and then incubated in rhodamine-conjugated goat antimouse IgG (H&L chain specific) (Hyclone, Logan, Utah) for 1 h at room temperature. Thereafter, the slides were washed with PBS, covered with Tris-glycerol, viewed with epifluorescence optics, and photographed.

RESULTS

Antibody Characterization

The specificity of both DM1B and Tu27b has been established previously. DM1B reacts with an epitope located within the C-terminal domain spanned by amino acids 416–431 [de la Viña et al., 1988]. The sequence of these 16 amino acids is entirely conserved in all avian and mammalian brain β-tubulin isotypes [Sullivan, 1988]. Although its binding site has not been identified, Tu27B also recognizes a highly conserved epitope [Caceres et al., 1984]. On immunoblots, this antibody reacts with β-tubulin in both vertebrate and invertebrate tissue extracts. Immunocytochemically, Tu27B stains all cell types in developing and adult neural tissue [Caceres et al., 1984]. Preliminary observations revealed that TuJ1 reacted only with β-tubulin in extracts of chicken brain and mammalian brain and testes [Frankfurter et al., 1986]. Additionally, both on immunoblots and in tissue sections, TuJ1 did not react with β-tubulin in glial cells [Trimmer et al., 1986]. These observations in conjunction with other reports [Sullivan et al., 1986; Burgoyne et al., 1988] indicated that TuJ1 is βIII-specific. We have used three independent methods to establish that the antibody binds only to the βIII C-terminus. First, because, β-tubulin isotypes can be distinguished by sequence differences within the last 15 C-terminal amino acids, we removed these isotype-defining domains from the β-tu-

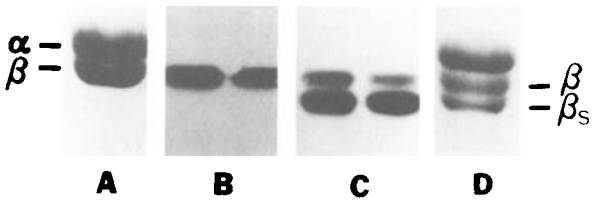

Fig. 1. Western blots of subtilisin digested purified rat brain tubulin. **A:** An Amido Black stained nitrocellulose strip showing undigested tubulin. **B:** A nitrocellulose strip showing subtilisn digested tubulin (45 and 60 min) stained with TuJ1. **C:** A nitrocellulose strip showing digested tubulin (45 and 60 min) stained with Tu27. **D:** An Amido Black stained nitrocellulose strip showing digested tubulin (30 min). α indicates α-tubulin subunit, β indicates undigested tubulin and β_s denotes the large proteolytic fragment (~48 kDa). Panel B demonstrates that the anti-βIII antibody TuJ1 does not react with β_s, indicating that the antibody reacts with an epitope located within the extreme C-terminal domain of the β-tubulin subunit.

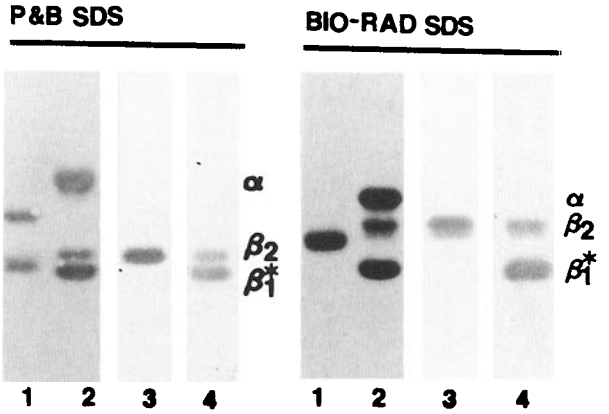

Fig. 2. Separation of EBI-crosslinked tubulin by SDS-PAGE. PC tubulin was crosslinked by EBI and S-carboxymethylated with 2-mercaptoethanol and iodoacetic acid. After crosslinking, the tubulin can be separated into three polypeptides: α-tubulin (α), EB1-crosslinked β-tubulin (β_1*), and uncrosslinked β-tubulin (β_2). The β_2 consists entirely of βIII. The two panels show Western blots of uncrosslinked and crosslinked tubulin separated in the presence of two different grades of SDS (see text). **Lane 1** in each panel shows the separation of uncrosslinked tubulin, and **lane 2** shows the separation of crosslinked tubulin (Amido Black stain). **Lane 3** in each panel shows that TuJ1 (anti-βIII) reacts only with the uncrosslinked β-tubulin (β_2). **Lane 4** in each panel shows that DM1B reacts with both the uncrosslinked and crosslinked β-tubulin.

bulin polypeptides with subtilisin. This protease preferentially cleaves a 1–2 kD peptide from the C-terminal end of the β-tubulin subunit [Sackett et al., 1985; de la Viña et al., 1988]. When the partially proteolyzed tubulin is separated by SDS-PAGE, α-tubulin, β-tubulin, and a β-tublin polypeptide (β_s ~ 48 kDa) lacking the isotype-defining C-terminal peptide(s) can be resolved. Immunoblots of the subtilisin digested protein show that TuJ1 reacts only with the residual unproteolyzed β-tubulin, whereas DM1B and Tu27 react with both the unproteolyzed and proteolyzed β-tubulin polypeptides (Fig. 1). Therefore, TuJ1 reacts with an epitope within the β-tubulin C-terminal isotype-defining domains. For a second approach to establish the specificity of TuJ1, we treated native tubulin with the irreversible sulfhydryl crosslinker, N,N′ethylene bis(iodoacetamide) (EBI). EBI treatment specifically produces an intra-β-subunit crosslink between cysteines 239 and 354 [Little and Ludueña, 1985]. All avian and mammalian brain β-tubulin isotypes, except βIII, possess cysteines at these two positions [Sullivan, 1988]. βIII has a serine for cysteine substitution at position 239. Consequently, this isotype is not affected by the crosslinking procedure. When reduced and S-carboxymethylated EBI-treated tubulin is separated by SDS-PAGE, the crosslinked β-tubulin migrates through the gel more rapidly than the uncrosslinked β-tubulin. The uncrosslinked β-tubulin consists entirely of βIII [Banerjee et al., 1988]. Figure 2 shows two sets of immunoblots with which we established the specificity of TuJ1. In both experiments, the antibody reacts exclusively with the uncrosslinked β-tubulin. The two sets of blots are shown to demonstrate that the composition of commercially available SDS markedly affects tubulin mobility. To optimize the separation of βIII, we use SDS which consists almost entirely of the C_{12} alkyl sulphate species. Third, to definitively establish the specificity of TuJ1 for βIII, we determined the reactivity of TuJ1 with bacterial fusion proteins containing β-tubulin C-terminal isotype-defining sequences. Bacterial fusion proteins containing the isotype-defining regions for chicken class II, III, IV, and V and human class I, II, III, IV, and V were separated by SDS-PAGE and Western blotted. TuJ1-immunoreacted blots reveal that the antibody only reacts with the fusion proteins containing the C-terminal sequences of βIII (Fig. 3). Additionally, the fact that TuJ1 does not bind to the tubulin in the chicken erythrocyte extract demonstrates that the antibody does not cross-react with chicken class VI (cβ6).

Expression and Tissue Distribution of βIII During Embryonic Development

To determine the onset of expression of $c\beta_4$, SDS extracts of either the heads or brains of chick embryos at stages 12, 14, 16, 18, and 23 were equated for the amount of total tubulin. Sample loadings were selected by densitometric scanning of DM1B stained immunoblots containing serial dilutions of each extract. Volumes yielding approximately equivalent intensity values within the linear range were chosen for further use. Immunoblots of normalized extracts stained with TuJ1 indicate that minimally detectable amounts of βIII are present as early as stage 12 (Fig. 4A). The ratios of intensity values obtained from densitometric scans of TuJ1 and DM1B

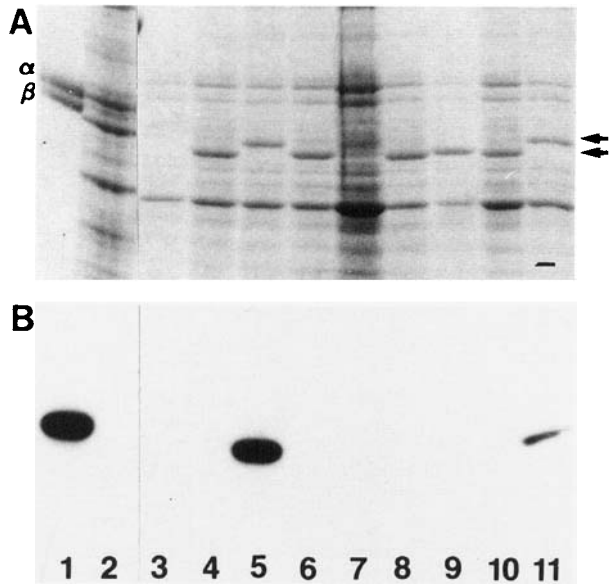

Fig. 3. Specificity of the monoclonal antibody TuJ1 for Class III β-tubulin isotype (βIII). **A:** Coomassive Blue stained gel: **lane 1** (rat PC tubulin); **lane 2** (chick erythrocyte extract); **lane 3** (control bacterial extract); **lanes 4–11** were loaded with bacterial extracts containing fusion proteins for chick class II (4), chick class III (5), chick class IV (6), chick class V (7), human class I (8), human class IV (9), human class II (10), and human class III (11). The arrows point to the fusion proteins. **B:** Immunoblot of a gel identical to that shown in A probed with TuJ1. The antibody reacts only with rat PC brain tubulin (**lane 1**) and the fusion proteins containing class III C-terminal sequences (**lanes 5** and **11**).

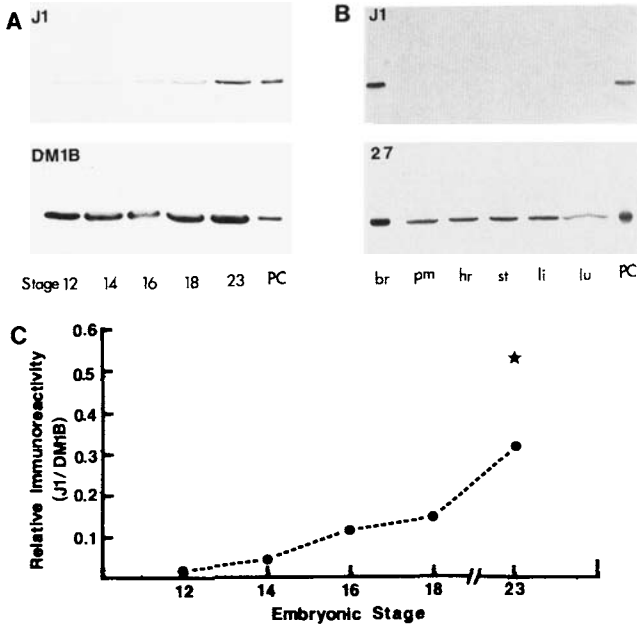

Fig. 4. Expression of $c\beta_4$ during embryonic brain development and $c\beta_4$ expression in different tissues. **A:** Whole SDS extracts of the heads of stages 12 (ca. 47 h incubation time), 14 (ca. 51 h), 16 (ca. 54 h), 18 (ca. 67 h), 23 (4 days) chick embryos were equated for total tubulin by DM1B staining. The volumes providing approximately equivalent DM1B intensity values by densitometric scanning were separated by SDS-PAGE, transferred to membranes, and the blots stained with either TuJ1 or DM1B. The blots show that there is gradual increase in the amount of βIII between stages 12 and 18. **B:** Total soluble protein from 14 day chick embryos was separated by SDS-PAGE (7.5% gel), transferred to membranes, and reacted with either TuJ1 or Tu27. TuJ1 reacts only with the brain extract and the rat PC brain tubulin, indicating that βIII is present only in neural tissue. Brain (br), pectoral muscle (pm), heart (hr), stomach (st), liver (li), lung (lu), rat brain tubulin (PC). **C:** Serial dilutions of the SDS extracts shown in A were separated by SDS-PAGE, transferred to membranes, and stained with either TuJ1 or DM1B. Both blots were densitometrically scanned and the J1/DM1B ratios, representing the amount of βIII relative to total tubulin, were calculated from dilutions giving intensity values in the linear range for both antibodies. The star (*) indicates the J1/DM1B ratio obtained for the day 14 chick embryo.

stained immunoblots reveal that the relative abundance of βIII increases linearly from stage 12 to stage 18. By stage 23, the relative abundance of the isotype has increased more than two fold (Fig. 4C). However, this increase is still approximately half the value calculated for the 14 day chick embryo. Although we did not establish when the relative abundance of βIII plateaus, it is evident that there is a marked induction of this protein between stages 18 and 23.

A comparison of Tu27B and TuJ1 stained immunoblots containing various organ extracts from 14 day chick embryos indicates that at this late stage in development, $c\beta_4$ expression is not detectable in non-neural tissues (Fig. 4B). Identical results were obtained with extracts obtained from hatchlings (data not shown).

βIII is a Substrate for Developmentally Regulated, Multiple-Site Posttranslational Modification

Numerous isoelectric focusing (IEF) studies have shown that brain tubulin becomes more heterogeneous with development [Denoulet et al., 1982; Wolff et al., 1982; Sullivan and Wilson, 1984; Eddé et al., 1989]. To determine the extent to which βIII contributes to this developmentally regulated increase in the number of β-tubulin isoforms, taxol-assembled brain microtubules from 14 day chick embryos and total brain protein from stage 18, 23, and day 14 embryos were separated by 2D-PAGE and transferred to membranes. DM1B-stained immunoblots of taxol-assembled microtubules from day 14 chick embryos separated by 2D-PAGE reveals that DM1B reacts with the same number of β-tubulin isoforms revealed by colloidal gold staining. In contrast, TuJ1 reacts only with a subset of these β-tubulin isoforms (Fig. 5A). Moreover, it is clear from the blots stained either with DM1B or colloidal gold that βIII resolves at a slightly higher apparent molecular weight than the other β-tubulin isoforms in the second dimension. This mobility difference is not unexpected since

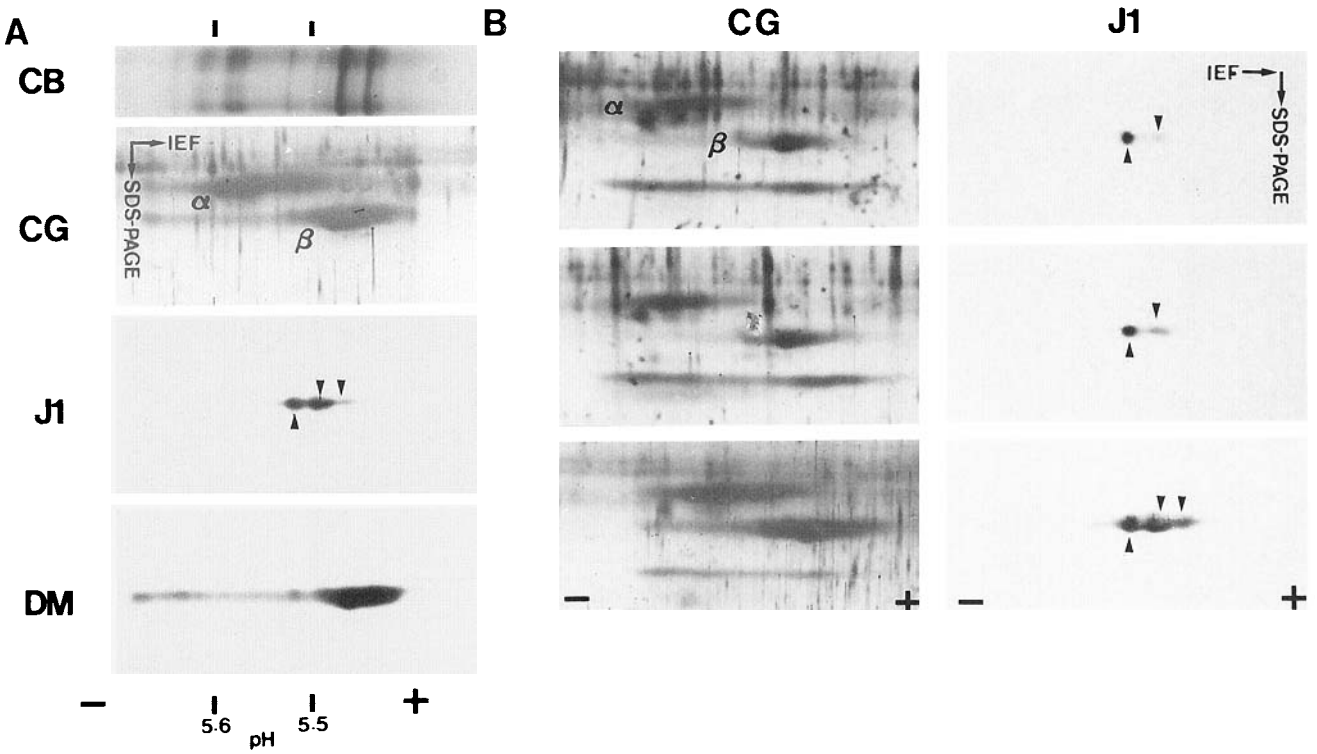

Fig. 5. βIII is a substrate for developmentally regulated, multiple site posttranslational modification. **A:** Taxol-assembled microtubules from the brains of 14 day chick embryos were separated by 2D-PAGE (7.5% gel), transferred to Immobilon P membranes, and the proteins stained with either colloidal gold (CG), TuJ1 (J1), or DM1B (DM). A Coomassie Blue (CB) stained lane from the IEF gel is shown at the top of the panel to indicate the location of the most prominent α-tubulin and β-tubulin isoforms (charge variants). A comparison of the three blots (CG, J1, DM) demonstrates that the anti-βIII antibody (TuJ1) reacts with three isoforms focusing in the most basic region of the pI range containing β-tubulin. **B:** Acetone precipitated proteins from SDS extracts of the heads of stage 18 and 23 chick embryos and the brains of 14 day chick embryos were separated by 2D-PAGE (7.5% gel), electrophoretically transferred to membranes, and the proteins stained with either colloidal gold (CG) or TuJ1 (J1). A comparison of the three immunostained blots shows that βIII becomes elephoretically heterogeneous with development. At stage 18, βIII resolves as a single isoform; whereas, at stage 23 and day 14 a second and then third isoform become immunologically detectable.

βIII contains more basic residues within its extreme C-terminal domain than the other chicken brain β-tubulin isotypes.

The blots of acetone precipitated protein from whole SDS extracts further demonstrate that βIII charge heterogeneity is developmentally regulated (Fig. 5B). From stage 18 embryo preparations, βIII resolves as a single basic spot. At stage 23, another, more acidic variant of βIII is present. By day 14, βIII can be resolved into at least three isoforms. Since βIII from different stage embryos was resolved in the same gel, it is extremely unlikely that the differences in charge heterogeneity are due either to modification during protein preparations or focusing.

Distribution of βIII in the Developing Nervous System

Most of the unequivocal TuJ1 immunoreactivity observed by immunofluorescence microscopy was localized to the nervous system at all embryonic stages. Within the nervous system, immunoreactive profiles were restricted primarily to regions containing post-mitotic neuroblasts and differentiating neurons. Intense immunofluorescence was associated only with the marginal zone of the neural tube at all levels of the neuraxis. Similarly, the cranial and peripheral ganglia were intensely immunofluorescent only when a definitive ganglionic structure was formed. Non-neural tissue (with minor exceptions), the neural crest, and migrating neural crest cells were unstained. No immunoreactive cells were observed at or before stage 12. The virtual absence of TuJ1 immunoreactivity in neuroblasts within the germinal zones and prior to stage 12 cannot be attributed to some methodologic artifact. Unequivocal staining with Tu27b was found in all cells at all stages. Only Tu27b was used as a control because DM1B does not react well with aldehyde-fixed tissue.

At all developmental stages, the extent and inten-

sity of TuJ1 immunoreactivity in the spinal cord coincided with the rostrocaudal development of the nervous system. More anterior portions of the spinal neural tube (SNT) contained a greater number of immunoreactive cells than more caudal regions (Fig. 6). A closer examination of less intensely stained more immature regions of the SNT revealed that most TuJ1 immunoreactive cells possessed processes and were located primarily at the marginal zone of the neural epithelium (Fig. 6A). The location and morphology of these cells suggests $c\beta_4$ expression is associated with post-mitotic neurons. Additionally, the majority of the early TuJ1-positive cells were located in the ventral lateral portion of the SNT. This observation is consistent with the earlier birth dates of larger motor neurons [Fujita and Fujita, 1963; Langman and Hayden, 1970].

TuJ1-immunoreactive bipolar cells with their soma located between the proliferative zone and the marginal zone were occasionally observed. The distal processes of these cells extended into the marginal zone of the neural tube (Fig. 6A). We believe these cells to be neuroblasts which have completed terminal mitosis shortly before sacrifice. Neuroblasts in this state of differentiation would be at this location as they withdraw from the proliferative zone following terminal mitosis and migrate toward the marginal zone of the neural tube. Qualitatively, these cells were less intense than cells within the marginal zone, suggesting that less βIII is present. The likelihood that $c\beta_4$ is expressed in mitotic neuroblasts as they traverse the neural epithelia cannot be ruled out; however, if this were the case, considerably more immunoreactive cells in the proliferative zones should be detected. Thus, the pattern of TuJ1 immunoreactivity in early stage SNT suggests that $c\beta_4$ expression is cotemporaneous with terminal mitotic division and that the isotype accumulates with further differentiation. More mature regions of the SNT show layers of immunoreactivity (Fig. 6D,E) consisting of neuronal cell bodies, intra-segmental fibers, and descending and ascending fibers of the spinal tracts. In all cases, a conspicuous absence of TuJ1 immunoreactivity was observed in the proliferative zone.

The staining pattern of developing dorsal root ganglia (DRG) by TuJ1 also indicates that $c\beta_4$ is expressed only by post-mitotic neurons in the peripheral nervous system. No TuJ1-immunoreactive cells were seen in areas associated with proliferating and migrating neural crest cells. In the anterior spinal regions of stage 17 embryos (Fig. 6A), a few bipolar cells with processes extending to the ventral horn and dorsal-lateral SNT were TuJ1-immunoreactive. This stage coincides with the time of initial appearance of differentiated neurons in the DRG [Hamburger and Levi-Montalcini, 1949], and the pattern of immunoreactivity at stage 17 and thereafter reflects the degree of neuronal differentiation. In the caudal spinal cord of stage 17 embryos (Fig. 6A), a few cells without any distinct morphology are weakly TuJ1 immunoreactive. These cells are likely to be neural crest cells that have just started to differentiate into DRG neurons. This observation again underscores the notion that the expression of $c\beta_4$ is coincident with terminal mitosis. The number of TuJ1 positive cells and fibers increase dramatically as additional neural crest cells differentiate and organize into the DRG. Also, as the sympathetic ganglia start to form, they become TuJ1-immunoreactive (Fig. 6D). In this figure, a pair of sympathetic ganglia have started to coalesce near the aorta.

The most prominent early TuJ1 immunoreactivity in the cranial region was in the trigeminal ganglion (Fig. 7A,B). The number of cells and the intensity of immunoreactivity increased with development of the ganglia. In some sections, placodal generation of neurons was observed before these cells have exited the placode (Fig. 7B). This observation is consistent with the report by Moody et al. [1989].

Expression of $c\beta_4$ during the development of the ciliary ganglion was also examined. The ciliary ganglion originates from cranial neural crest [Narayanan and Narayanan, 1978] and is a relatively late developing ganglion. The earliest TuJ1 immunoreactivity in the ciliary ganglia occurred at or near stage 22 (Fig. 7C). This initial staining pattern reveals TuJ1 positive cells distributed sparsely and randomly around the oculomotor nerve. At stage 25, when cell proliferation has ceased [Pilar and Tuttle, 1982], there was a dramatic increase in the number of immunoreactive cells (Fig. 7D). Additionally, the immunoreactive cells were more compactly organized and the post-ganglionic nerve fibers extending along the medial surface of the optic cup are well delineated by TuJ1 staining. Both the trigeminal and ciliary ganglia are small structures with relatively few neurons, and their development has been studied in detail. Consequently, it is possible in these cell groups to precisely correlate the appearance of TuJ1 immunoreactivity with the cessation of mitotic activity and the initiation of differentiation.

As in the SNT, most of the TuJ1 immunoreactivity in the retinal (Fig. 8) and cranial (Fig. 9) neural tubes was associated with neuroblasts in the marginal zone of the neural epithelium. In the developing retina, ganglion cells were TuJ1 immunoreactive as early as stage 17 (Fig. 8A). The immunoreactive axons could be followed out of the retina to the growth cone-like tips (Fig. 8C). The increase in the number and the intensity of TuJ1-immunoreactive cells in the cranial neural tube corresponds well with the increase in relative abundance of βIII documented in Figure 4. In some of the sections, cells were occasionally observed with low but definite

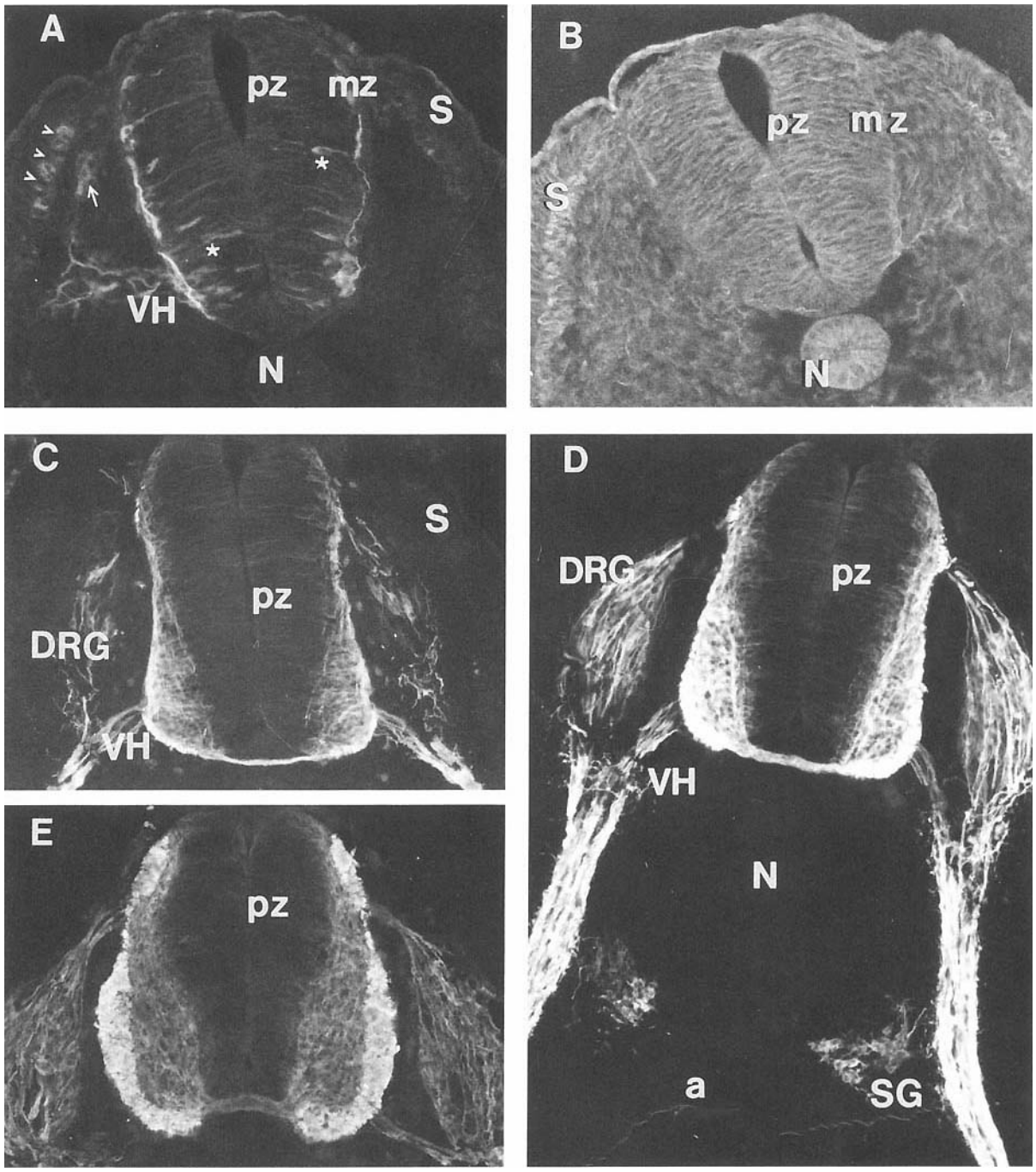

Fig. 6. The expression of $c\beta_4$ in the developing spinal cord. Cryostat sections of spinal cord stained with TuJ1 (A, C, D, and E) and Tu27B (B). **A** and **B:** Caudal spinal cord at stage 18. **C:** Thoracic spinal cord at stage 18. **D:** Thoracic spinal cord at stage 22. **E:** Thoracic spinal cord at stage 25. At every stage, most of the TuJ1 immunoreactive cells are confined to the marginal zone (mz). Immunoreactive cells were never detected in the neural crests. The neural crest derived ganglion neurons do not become immunoreactive until they accumulate in the coalescing ganglia. In the least developed spinal cord shown (A), a small number of weakly immunoreactive spindle-shaped cells (*) and their processes can be located in the intermediate zone of the neural epithelium. The location and morphology of these cells suggests that $c\beta_4$ is expressed during or immediately following the final mitotic cycle. To underscore this point, a few putative dorsal root ganglion (DRG) cells are TuJ1 immunoreactive (arrow in A) well before other ganglion cells have differentiated (compare A and C). Thus, the expression of this tubulin isotype may be one of the earliest indicators of neuronal differentiation. Also shown are TuJ1-immunoreactive cells in the somite (arrowheads in A). The expression of $c\beta_4$ in the somites appears to be transient since no comparable immunoreactivity is seen in more developed spinal areas. N, notocord; S, somite; VH, ventral horn; SG, sympathetic ganglion; a, aorta, pz, proliferating zone.

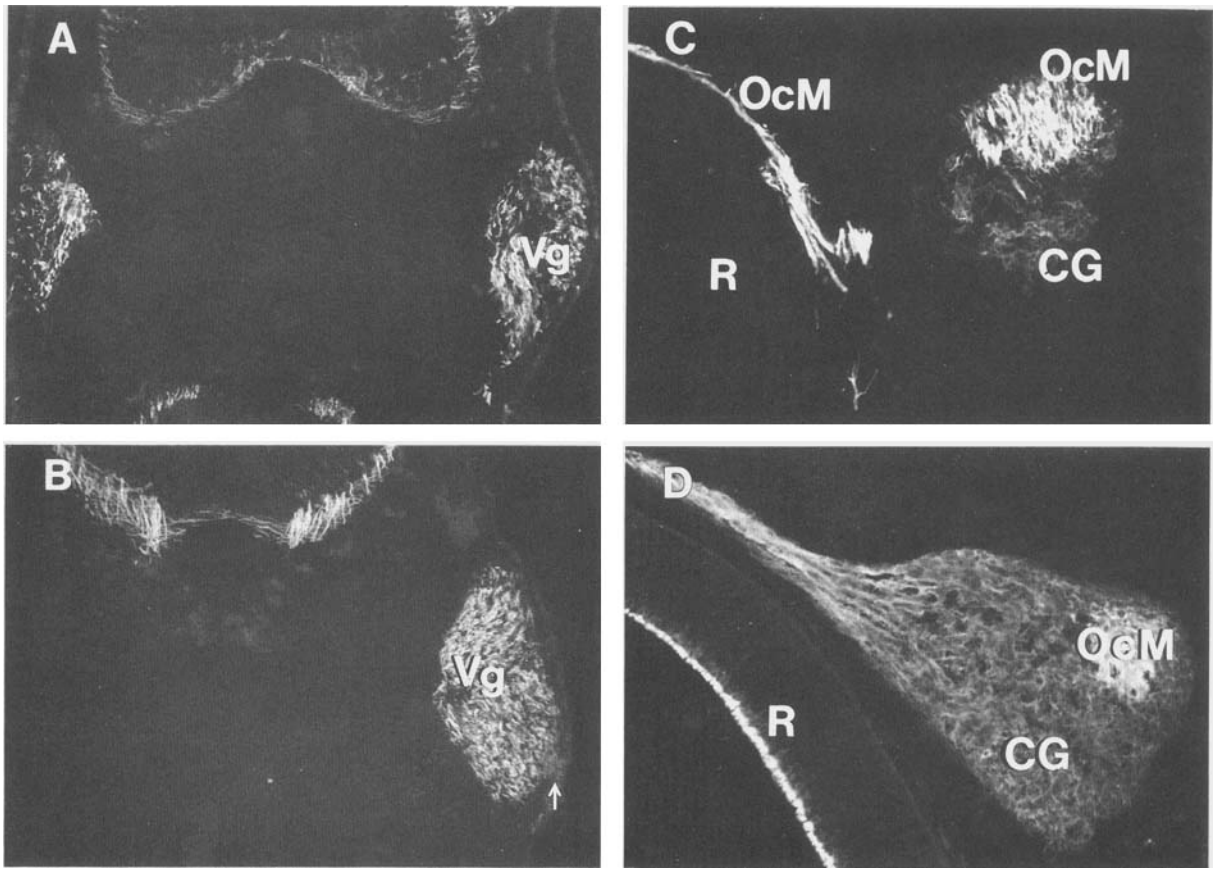

Fig. 7. Expression of $c\beta_4$ in the trigeminal and ciliary ganglia. TuJ1 stained sections through the trigeminal ganglia (Vg) at stage 17 (**A**) and at stage 22 (**B**). The number of βIII positive cells increases with development. Note the generation of ganglionic cells from the placode (B, vertical arrow). **C** and **D:** Sections through optic cup stained with TuJ1 showing the ciliary ganglia (CG). At stage 22 (C) TuJ1 immunoreactive CG cells are sparce. At stage 25 (D), as the CG has formed around the oculo-motor nerve (OcM), TuJ1 immunoreactivity increases. (R) retina.

TuJ1 immunoreactivity in the proliferative, ventricular zone (Fig. 9B). These cells were often present in pairs, suggesting that they are clonally related pairs of neuroblasts which recently have completed their final mitotic division.

The expression of $c\beta_4$ in the cranial nervous system, as in the spinal and peripheral nervous system, is clearly associated with terminally differentiating neurons. Taken in sum, these observations indicated that βIII is present in all neurons during differentiation but not in undifferentiated neural precursors. Thus, the presence of βIII is an early marker for postmitotic neurons, fibers, and growth cones.

Non-Neural TuJ1 Immunoreactivity

While immunoreactivity in the nervous system was restricted to neurons, staining was present in a small number of other tissues. The embryonic lens exhibited low but specific immunoreactivity. Transient but intensely TuJ1 positive cells were seen in the caudal mesonephric duct (Fig. 10A) and the caudal-ventral somites (Fig. 6A) of earlier stage embryos (17/18). In the mesonephric duct, TuJ1 immunoreactivity was not detectable in later stage embryos (data not shown). Similarly, TuJ1-immunoreactive cells could not be detected in the somites of more developed spinal areas (see Fig. 6). Intense TuJ1-immunoreactive cells were also present in the amion. If the embryonic membranes are removed prior to sectioning, immunoreactivity at the surface of the embryo is lost. The position of the membrane containing the TuJ1 positive cells when viewed with differential interference contrast microscopy indicates that these cells are ectodermal cells of the amnion (Fig. 10B,C).

DISCUSSION

The studies described in this report indicate that the onset of $c\beta_4$ expression is contemporaneous with the

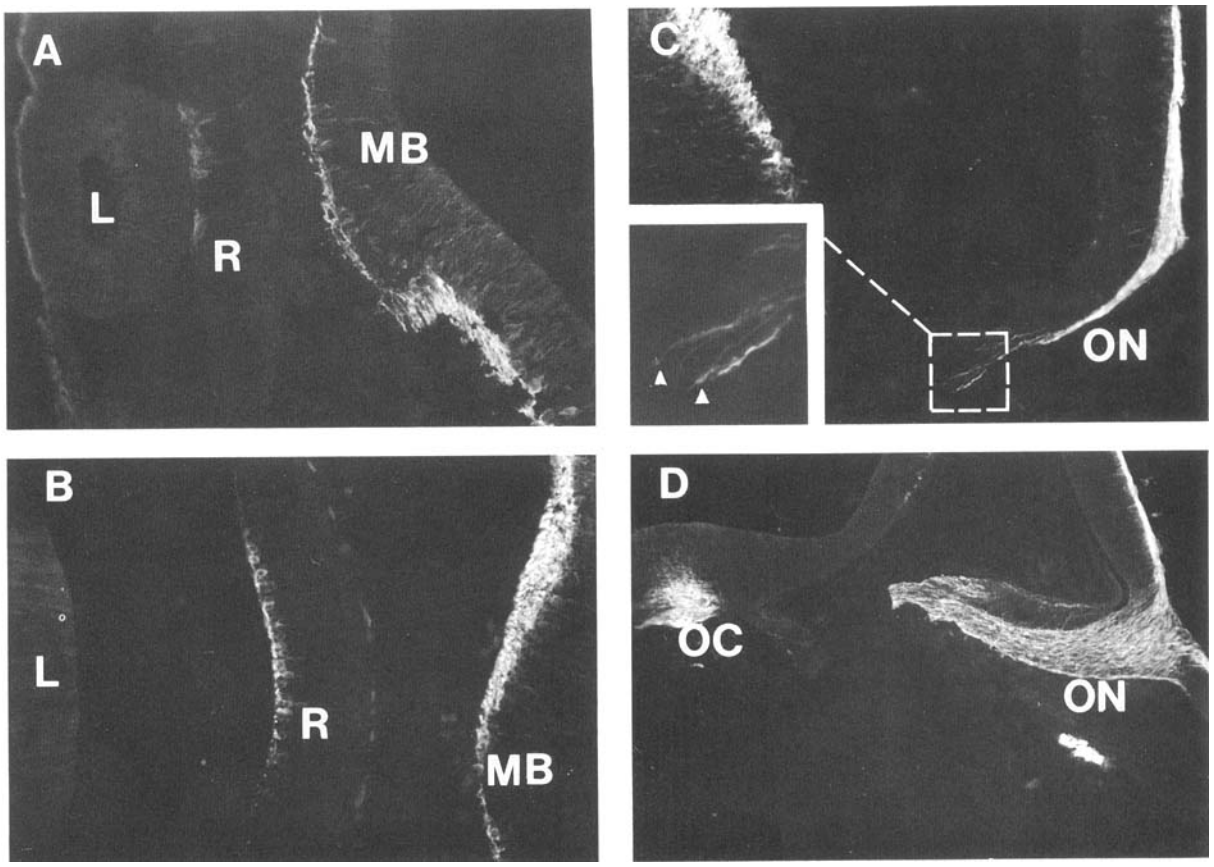

Fig. 8. Expression of $c\beta_4$ in retinal ganglion cells (**A**) and (**B**). Sections through the optic cup near the mid-brain (MB) show that TuJ1-immunoreactive cells are beginning to appear in the retina (R) at stage 18 (A) as the lense vesicle (L) contacts the retina. These are optic ganglion cells which will give rise to the optic nerve. At stage 22 (B), the cells have assumed a laminar organization and the intensity of the staining has increased. Note also that most of the TuJ1 immunoreactivity in the midbrain is confined to the marginal zone and that some TuJ1 immunoreactivity is associated with the lens at stage 22. **C** and **D:** Sections through the optic stalk at stage 22 (C) and at stage 25 (D) stained with TuJ1. The **inset** in C shows an enlargement of the boxed area. Note the immunoreactivity associated with some optic fiber growth cones (arrowheads). MB, midbrain; OC, optic chiasm; ON, optic nerve; R, retina.

earliest phase of neuronal differentiation. The complete absence of staining in the neural crest, as well as the location, morphology, and pairing of very lightly stained cells in the proliferative zone of the neural epithelium suggests that the expression of this isotype commences immediately after neuroblasts complete their final mitotic division. This interpretation is further supported by the fact that the increase in relative abundance of βIII between stages 12 and 23 coincides with the accumulation of terminally differentiated neurons. Consequently, the expression of $c\beta_4$ may be one of the earliest indications of neuronal commitment.

Although transiently expressed in a very small number of non-neural structures, our studies confirm previous observations that $c\beta_4$ expression is entirely neuron-specific at later stages of development [Burgoyne et al., 1988]. In this regard, $c\beta_4$ differs from that of its mammalian homologues since in mammals βIII is also present in the testes [Frankfurter et al., 1986; Lewis and Cowan, 1988].

The isoelectric focusing experiments conclusively establish that βIII is a substrate for developmentally regulated, multiple-site posttranslational modification. The identification of at least three isoforms, indicating that the isotype is subject to multiple modifications was not expected. The results from several studies collectively indicate that in brain βIII is the only isotype phosphorylated and that the modification occurs exclusively on the C-terminal serine at residue 444 [Ludueña et al., 1988]. In view of this evidence, we expected to resolve two βIII isoforms, the most basic variant representing the unmodified protein, and the more acidic variant representing the phosphorylated protein. Indeed, the difference in pI between the most basic isoform and the second more acidic isoform can be explained as the addition of a single phosphate. Clearly, the subsequent appearance of a third most

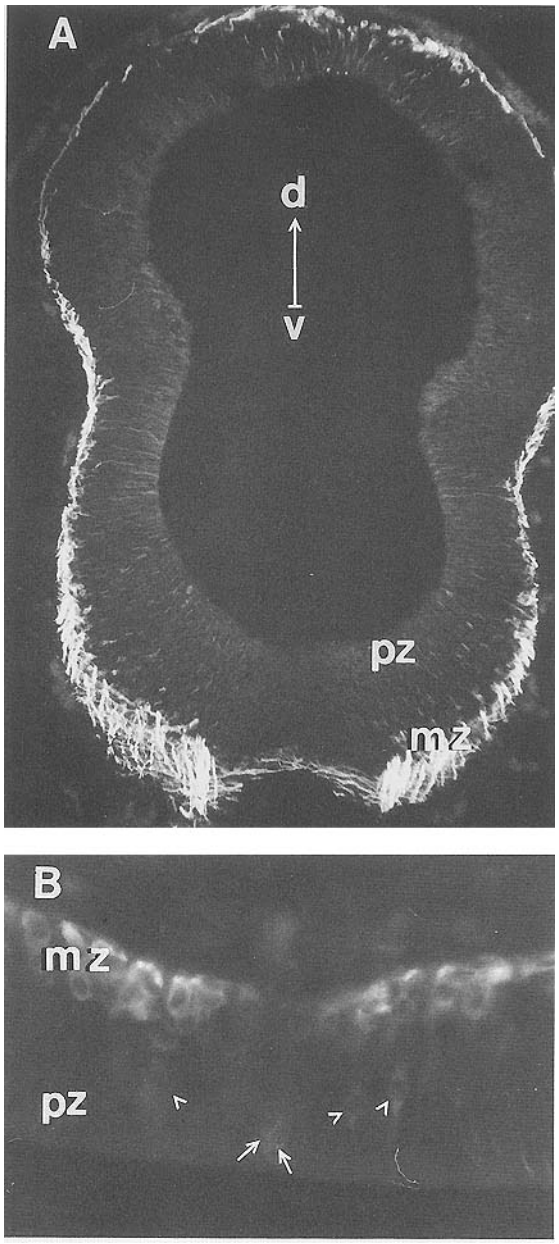

Fig. 9. Expression of $c\beta_4$ in crainal neural epithelium. **A:** A cryostat section through the neural epithelium of a stage 22 chick embryo stained with TuJ1. The figure shows a coronal section through the mesencephalon. Note, as in the spinal cord, most of the staining is limited to the marginal zone (mz). **B:** A higher magnification of the area analogous to the dorso-midline region shown in A. Note the pair of cells with low immunoreactivity in the proliferating zone (arrows) and the immunoreactive cells in the intermediate zone (arrows) and the immunoreactive cells in the intermediate zone (arrowheads). These observations reinforce the suggestion that class III β-tubulin expression is coincident with neuronal commitment.

acidic isoform cannot be predicted from a single site phosphorylation event. By isoelectric focusing of proteolytically digested tubulin we have determined that this second posttranslational modification is also located within the isotype-defining domain [Lee et al., 1990]. Although the βIII isotype-defining domain contains a potential phosphorylation site on the tyrosine at residue 437, and while we cannot completely rule out the possibility that this residue is phosphorylated, it appears unlikely since there is no evidence indicating that in vivo any β-tubulin isotype is a substrate for tyrosine phosphorylation. In any event, we consider it highly significant that the C-terminal domain becomes increasingly negatively charged during development. It is thought that the extreme C-terminus of the β-tubulin polypeptide regulates microtubule assembly through its interaction with microtubule-associated proteins (MAPs) [Serrano et al., 1984; Sackett et al., 1985; Littauer et al., 1986], presumably by electrostatic interaction [Joly et al., 1989]. If this contention is correct, then the modified C-terminal domains should bind MAPs more efficiently than the unmodified ones, resulting in either more rapid assembly or greater microtubule stability.

Our results also suggest that during the initial phase of neural differentiation much of βIII is either not stably incorporated into microtubules or does not efficiently assemble into microtubules. This interpretation is based on the following observations. At stage 18 βIII resolves as a *single* basic isoform (the unmodified isotype). In a previous study, Gard and Kirschner [1985] showed by isoelectric focusing that βIII phosphorylation in neuroblastoma occurred only after the isotype was incorporated into microtubules. Nocodazole-induced depolymerization resulted in massive dephosphorylation, and the subsequent removal of the drug again increased the amount of incorporated ^{32}P. Similarly, taxol-stimulated microtubule assembly increased the level of phosphorylated βIII. These results suggest that the extent of phosphorylation is an *indication* of how much βIII is stably incorporated into microtubules and also indicates that phosphorylation is not required for the isotype to coassemble into microtubules. The fact that only a single βIII isoform can be detected in SDS extracts of stage 18 embryos indicates that there is little, if any, posttranslationally modified βIII present at this time in development. Our interpretation is consistent with the results from two other previous studies which have shown that during NGF-stimulated neurite outgrowth by PC12 cells less βIII is incorporated into microtubules than some other β-tubulin isotypes [Asai and Remolona, 1989; Joshi and Cleveland, 1989].

It is also noteworthy that the depletion of βIII from purified bovine brain tubulin by immunoaffinity chromatography results in an increase in the rate and extent of MAP-stimulated microtubule assembly [Banerjee et al., 1990]. This result suggests that even the adult posttranslationally modified isoforms of βIII do not assemble into

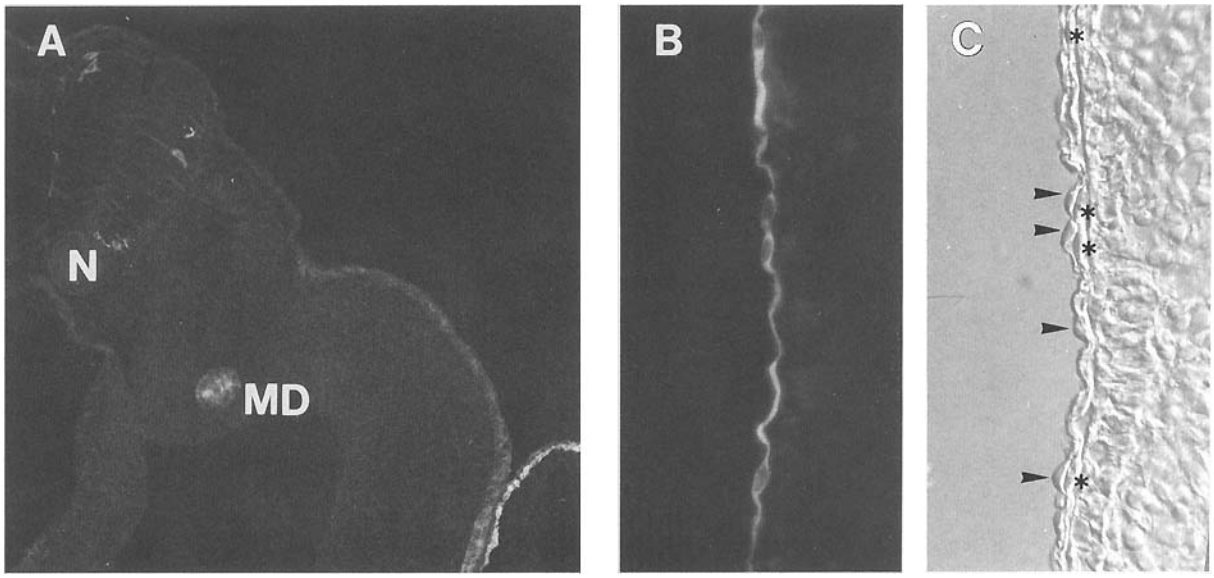

Fig. 10. Non-neural TuJ1 immunoreactivity. **A:** A section through the lumbo-sacral region stained with TuJ1. Note the intense TuJ1 immunoreactivity in the mesonephric duct (MD). **B** and **C:** TuJ1 immunoreactivity in the amnion (B) and a corresponding DIC image (C). Note that only the cells in the ectodermal layer are immunoreactive to TuJ1 (*), while the endodermal cells (arrowheads) are TuJ1-negative.

microtubules as efficiently as other β-tubulin isotypes and may actually retard the polymerization process. Whether or not, and how, the sequential posttranslational modification of βIII modulates the interaction between MAPs and tubulin clearly needs answering.

In summary, βIII differs by approximately 10% in amino acid sequence from other β-tubulin isotypes present in avian and mammalian brain. The isotype contains a unique serine phosphorylation site within its C-terminal isotype-defining domain and a serine for cysteine substitution at residue 239. This substitution appears to be a conserved feature of all chordate neural-specific β-tubulin isotypes [Ludueña et al.,1982]. The appearance of βIII coincides with the terminal mitosis of neuroblasts. This event is also contemporaneous with the extension of permanent neurites: the axons and dendrites. Consequently, the expression of $c\beta_4$ in the nervous system also coincides with the appearance of cellular structures that are unique to neurons. This correlation suggests that the functional properties of βIII are adapted to the unusual functional requirements of the neuronal cytoskeleton. During development βIII is subject to temporally regulated, multiple-site posttranslational modification within its isotype-defining domain. This observation and the fact that βIII exhibits distinctive properties both in vitro and in vivo reinforces the idea that this isotype is an important, perhaps essential, regulatory component of the neuronal cytoskeleton.

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