In Vivo Coassembly of a Divergent β-Tubulin Subunit (cβ6) into Microtubules of Different Function

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Abstract. α- and β-Tubulin are encoded in vertebrate genomes by a family of ~6–7 functional genes whose polypeptide products differ in amino acid sequence. In the chicken, one β-tubulin isotype (cβ6) has previously been found to be expressed only in thrombocytes and erythroid cells, where it is assembled into a circumferential ring of marginal band microtubules. In light of its unique in vivo utilization and its divergent assembly properties in vitro, we used DNA transfection to test whether this isotype could be assembled in vivo into microtubules of divergent functions. Using an antibody specific to cβ6, we have found that upon transfection this polypeptide is freely coassembled into an extensive array of interphase cytoplasmic microtubules and into astral and pole-to-chromosome or pole-to-pole microtubules during mitosis. Further, examina-

tion of developing chicken erythrocytes reveals that both β-tubulins that are expressed in these cells (cβ6 and cβ3) are found as co-polymers of the two isoforms. These results, in conjunction with efforts that have localized various other β-tubulin isotypes, demonstrate that to the resolution limit afforded by light microscopy in vivo microtubules in vertebrates are random copolymers of available isotypes. Although these findings are consistent with functional interchangeability of β-tubulin isotypes, we have also found that in vivo microtubules enriched in cβ3 polypeptides are more sensitive to cold depolymerization than those enriched in cβ6. This differential quantitative utilization of the two endogenous isotypes documents that some in vivo functional differences between isotypes do exist.

M ICROTUBULES are ubiquitous structural components of eukaryotic cells. In all dividing cells, they comprise the major components of mitotic spindles and in concert with actin filaments and intermediate filaments, they establish the internal cytoarchitecture of the interphase cell cytoplasm (e.g., Heuser and Kirschner, 1980). In addition, microtubules function in several specialized cell processes including meiosis, flagellar dependent cell motility, elongation of neurites, the transport of vesicles through the cytoplasm, and assembly of a circumferential ring of microtubules in hematopoietic cells.

Although the principal subunit of each of these different classes of microtubules is a heterodimer of one α- and one β-tubulin polypeptide, each subunit is encoded in vertebrates by a family of ~6–7 functional genes (Lopata et al., 1983; Hall et al., 1983; Lewis et al., 1985a, b; Wang et al., 1986; Sullivan et al., 1986a, b; Elliott et al., 1986; Villasante et al., 1986; Pratt et al., 1987). The β-tubulin polypeptides produced from these genes have distinct amino acid sequences and are distinguished one from another principally by sequences within a carboxy-terminal ~15 amino acid-variable region domain (Sullivan and Cleveland, 1986). Recently, we cloned and sequenced the major β-tubulin subunit that is assembled into marginal band microtubules in nucleated erythrocytes and thrombocytes of chickens (Murphy et al., 1987). Like a mammalian β-tubulin that is assembled into marginal

bands in mouse platelets (mβ1; Wang et al., 1986; Lewis et al., 1987), cβ6 tubulin is expressed exclusively in cells that assemble marginal bands and its sequence diverges from other chicken β-tubulins in nearly one fifth of the residue positions. This relatively low homology stands in marked contrast to the much smaller divergence in the sequences of the remaining six chicken β-tubulin polypeptides (which show sequence identity in 92–99% of amino acid positions).

The existence within a single organism of a group of distinct isotypes of α-tubulin and β-tubulin has encouraged speculation that divergent functional classes of microtubules are specified, at least in part, by their assembly from different isoforms (Fulton and Simpson, 1976; Stephens, 1975; see Cleveland, 1987 for review). This hypothesis has been supported by the identification of evolutionarily conserved isotypes of β-tubulin whose primary sequence and patterns of expression have been very highly conserved (Sullivan and Cleveland, 1986).

Nonetheless, genetic and biochemical evidence from *Drosophila* has demonstrated that at least one β-tubulin is multifunctional and is used to construct all classes of microtubules during spermiogenesis (Kemphues et al., 1982; Raff, 1984; Fuller et al., 1987). Similarly, Solomon and co-workers have shown that a chimeric β-tubulin composed of an amino terminus of chicken β2-tubulin linked to a divergent carboxy terminus from a yeast β-tubulin is incorporated into all

classes of mouse fibroblast microtubules that are distinguishable by light microscopy (Bond et al., 1986). Further, Swan and Solomon (1984) have demonstrated in chicken erythrocytes that the divergent cβ6 polypeptide can be replaced with brain isotypes during in vitro reassembly and Cowan's group has shown that the hemopoetic-specific mβ1 subunit is incorporated into multifunctional classes of microtubules when expressed in HeLa cells (Lewis et al., 1987). These experiments offer strong evidence for the functional equivalence of β-tubulin subunits.

The identification of a cDNA clone containing the entire coding sequence of cβ6, in conjunction with antibodies specific to each of the six β-tubulin isotypes expressed in chicken (Lopata and Cleveland, 1987; Murphy et al., 1987), has allowed us to reinvestigate tubulin isotype utilization. By expression of cβ6 transfected into cells in which it is not normally found, we have now analyzed whether this divergent vertebrate tubulin can be assembled in vivo into "inappropriate" microtubules. We have found that this cβ6 tubulin is coassembled into all classes of microtubules. Similarly, we have used antibodies specific to individual isotypes to demonstrate that, even in its normal erythrocyte environment, cβ6 is coassembled with cβ3 into developing marginal bands in immature erythrocytes and into marginal bands of terminally differentiated cells. In conjunction with our companion efforts (Lopata and Cleveland, 1987) and those of Cowan and collaborators (Lewis et al., 1987), these studies demonstrate that in vivo cellular microtubules in higher eukaryotes are assembled as copolymers from available isoforms. However, our additional finding that microtubules enriched in cβ3 are less stable to cold-induced depolymerization reveals that in vivo some functional distinctions among tubulin isotypes must be present.

Materials and Methods

Cell Culture and Transfection

COS, a line of monkey kidney cells that constitutively express SV40 T antigen (Gluzman, 1981), were grown on plastic dishes in Dulbecco's modified Eagle's medium (DME) plus 10% fetal calf serum. Cells were transfected either using plasmid DNA complexed to DEAE-dextran (Lopata et al., 1984) or by the calcium phosphate coprecipitation method (Graham and van

der Eb, 1972) with addition of a chloroquine shock step (Luthman and Manussion, 1983). 24 h posttransfection, cells were trypsinized and replated onto glass coverslips for immunofluorescence observations. Cells were analyzed between 36 and 72 h posttransfection.

Antibodies Used

A rabbit polyclonal antibody specific for the chicken cβ6 isotype was provided by Dr. D. Murphy (of Johns Hopkins University, Baltimore, MD) (Murphy et al., 1986). To remove cross-reactivity to other β-tubulin isotypes, the cβ6 antiserum was affinity-purified on a column of chicken brain tubulin covalently linked to Sepharose. Antibodies that did not bind to the column were recovered and were utilized in the cβ6 experiments. Prior to use in immunofluorescence against COS cells, the antibody was adsorbed against fixed COS cytoskeletons attached to culture dishes.

A rabbit polyclonal antibody specific to chicken isotype cβ3 (isotypic class c-IV; see Table I and Lopata and Cleveland, 1987) was prepared by covalently cross-linking the carboxy-terminal 9 amino acids of cβ3 to keyhole limpet hemocyanin followed by injection into rabbits. The characterization of this antibody is given in Lopata and Cleveland (1987). All cβ3 antibodies were affinity-purified by chromatography of the cβ3-derived antiserum on an affinity column of cβ3 carboxy-terminal peptide linked to Sepharose. Precise conditions of the chromatography are given in Lopata and Cleveland (1987).

Antibodies specific for β-tubulin isotypes I, II, III, and c-V were also generated using peptide antigens corresponding to the extreme carboxy-terminal amino acids (peptides of length between 9 and 17 residues were used). Each of these (except antibody to type III) was affinity-purified as described earlier (Lopata and Cleveland, 1987).

Monoclonal antibody (3B3) that recognizes β-tubulin was provided by Dr. Steve Blose (Protein Databases, Inc., Huntington Station, NY). Immunoblotting with cloned fusion proteins for each β-tubulin isotype (like that shown in Fig. 4 B) demonstrated that this antibody binds to all β-tubulin isotypes (Lopata, M. A., and D. W. Cleveland, unpublished observation). A second monoclonal antibody that recognizes α-tubulin was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY).

To demonstrate specificity of the anti-cβ3 and anti-cβ6 antibodies, cloned fusion proteins consisting of the amino-terminal 32 kD of the bacterial trpE protein linked to the ~100 carboxy-terminal amino acid residues from either cβ6 or cβ3 were expressed in bacteria as described earlier (Lopata and Cleveland, 1987). Bacterial lysates containing induced fusion proteins were analyzed by protein immunoblotting.

Indirect Immunofluorescent Localization of Tubulin Isoforms

For visualization of microtubules in transfected COS cells, cells were trypsinized after transfection and replated onto glass coverslips. After attachment and growth overnight, the coverslips were washed for 15 s at 37°C with stabilization buffer (0.1 M Pipes [pH 6.9], 1 mM EGTA, and 4 M glycerol [Solomon et al., 1979; Osborn and Weber, 1982]) and then incubated for

Table I. Chicken β-Tubulin Genes and Isotypes

Gene	Polypeptide isotype*	Carboxy-terminal isotypic sequence‡	Pattern of expression
cβ1	II	DEQGEFEEEGEEDEA	many tissues; major gene in muscle§
cβ2	II	DEQGEFEEEGEEDEA	many tissues; major brain isotype§
cβ3	c-IVǁ	EEEGEFEEEAEEEAE	many tissues; dominant testis isotype§
cβ4	III	EEEGEMYEDDEEESEQGAK	neuronal specific; minor brain isotype¶
cβ5	c-V	NDGEEAFEDDEEEINE	almost all cell types examined except neurons**
cβ6	—‡‡	DVEEYEEAEASPEKET	thrombocytes and erythrocytes§§
cβ7ǁǁ	I¶¶	EEEEDFGEEAEEEA	constitutively§

* Sequences begin at amino acid 431.
‡ Isotypic classes defined by evolutionarily conserved carboxy-terminal region sequence and pattern of expression; Sullivan and Cleveland, 1986.
§ Havercroft and Cleveland, 1984.
ǁ Identified as chicken type IV in Lopata and Cleveland, 1987.
¶ Sullivan and Cleveland, 1984.
** Sullivan et al., 1986a.
‡‡ No evolutionarily conserved sequence has been identified in other organisms.
§§ Murphy et al., 1987.
ǁǁ Originally referred to as cβ4'—Havercroft and Cleveland, 1984.
¶¶ Monteiro, M. J., and D. W. Cleveland, unpublished observations.

1 min at 37°C in stabilization buffer containing 0.5% Triton X-100. Coverslips were then rinsed in stabilization buffer (15 s at 37°C) and plunged into methanol at −20°C for 5 min. Coverslips were rehydrated in phosphate-buffered saline (PBS) and stained either with the cβ6-specific antibody (diluted 1:20 in PBS) or a β-tubulin monoclonal antibody for 1 h at room temperature. They were then rinsed with PBS and either a fluorescein-labeled anti–rabbit IgG antibody or a fluorescein-labeled anti–mouse IgG antibody (Cooper Biomedical Inc., Malvern, PA) was applied for 45 min at room temperature. The coverslips were rinsed with PBS and mounted in Aquamount (Lerner Laboratories, New York). The cells were examined on an Olympus BH2 microscope with epifluorescence optics and photographed on Kodak Tri-X film (Eastman Kodak Co., Rochester, NY) developed in Diafine.

For simultaneous localization of total tubulin and of the cβ6 isotype in microtubules of transfected cells, coverslips containing transfected cells were first stained with the anti–cβ6 antibody (as outlined above except that a secondary antibody bound to rhodamine was used) and then stained with the 3B3 monoclonal antibody that recognizes all β-tubulin isotypes (Lopata, M. A., and D. W. Cleveland, unpublished observations). Binding of the monoclonal antibody was then detected with a fluorescein-labeled goat anti–mouse IgG (Cooper Biomedical, Inc.).

For immunofluorescent localization of cβ3 and cβ6 isotypes in mature chicken erythrocytes, blood was obtained from an adult chicken by wing vein puncture. 200 µl of blood was diluted into 5 ml of citrate-saline (0.3% sodium citrate, 0.9% NaCl, pH 7.4), and centrifuged at 1,000 g for 3 min. The supernatant and buffy coat were removed by aspiration. Cells were washed twice with PBS at 37°C. Glass coverslips were coated with 0.1% poly-L-lysine and fixed in 1% glutaraldehyde and washed extensively with water. The polylysine-coated coverslips were then overlaid for 5 min with 5 ml of erythrocyte cell suspension in PBS at 37°C. Coverslips containing attached cells were used immediately for immunostaining following the protocol details above. For analyzing cold stability of erythrocyte microtubules, coverslips with attached cells were incubated at 4, 10, or 20°C for up to 4 h in PBS before immunostaining.

Production of Cloned Fusion Proteins for Each Chicken β-Tubulin Isotype

Bacterial extracts containing cloned fusion proteins carrying the carboxy-terminal sequences corresponding to four chicken β-tubulin isotypes (type II [encoded by genes cβ1/cβ2], type III [encoded by gene cβ4], type c-IV [encoded by gene cβ3], and type c-V [encoded by gene cβ5]) were prepared as described earlier (Lopata and Cleveland, 1987). For type I β-tubulin (encoded in chicken by gene cβ7 [originally called cβ4'; Havercroft and Cleveland, 1984]), the carboxy-terminal sequence is identical to that previously determined for mammalian type I (Monteiro, M. J., and D. W. Cleveland, unpublished); consequently, we utilized the human type I fusion protein previously described (Lopata and Cleveland, 1987). For each tubulin isotype, the actual fusion protein was composed of an amino-terminal 32 kD of the bacterial protein trpE linked to the β-tubulin isotype sequence beginning at residue 345 and continuing through to the normal carboxy terminus of the β-tubulin isotype.

For production of a fusion protein to cβ6, a plasmid containing a nearly full-length copy of cβ6 mRNA (clone 8; Murphy et al., 1987) was digested with Bam HI (which cut within codon 344 and at a site in the 3' untranslated region sequence). The 507-base fragment containing cβ6 carboxy-terminal coding sequences was then ligated to the Bam HI site that lies 32 kD into the trpE gene in plasmid pATH 1. A clone containing the cβ6 sequence in the proper orientation was obtained and expression was induced as before (Lopata and Cleveland, 1987).

Gel Electrophoresis and Immunoblotting

Polyacrylamide gels for analysis of protein samples were run as described by Laemmli (1970). Proteins were transferred to nitrocellulose (BA83, Schleicher & Schuell, Inc., Keene, NH) in one-half strength Laemmli gel running buffer (Laemmli, 1970) containing 20% methanol. Typically, transfer was for 4 h at 50 V. Filters containing transferred samples were stained with Ponceau S (0.2% Ponceau S in 3% trichloroacetic acid) to identify positions of molecular weight standards. Nonspecific protein binding sites were blocked by incubation in 4% bovine serum albumin (BSA) in phosphate-buffered Triton X-100 (PTX) (0.2% Triton X-100, 0.15 M NaCl, 10 mM sodium phosphate [pH 7.5], 1 mM EGTA) for 10 min. For immunologic detection of proteins, primary antibody in PTX containing 4% BSA was then allowed to react overnight at room temperature. Unbound primary

antibody was removed and the filters were washed five times (3 min each wash) in wash buffer (WB) (0.5% Triton X-100, 50 mM triethanolamine [pH 7.4], 0.1 M NaCl, 0.1 mM EDTA, 1 mM SDS). ^{125}I-labeled protein A was added and incubated for 1 h at room temperature, followed by five washes (3 min each wash) in WB to remove unbound protein A. Binding was detected by autoradiography using Dupont Lightning Plus intensifying screens (DuPont Co., Wilmington, DE) and Kodak XAR film.

Analysis of Soluble and Polymeric Levels of cβ6 and cβ3 in Populations of Adult Erythrocytes

Mature erythrocytes were prepared from adult blood obtained by wing vein puncture. Cells were pelleted in a clinical centrifuge, resuspended in citrate-saline (0.9% NaCl, 0.3% Na-citrate), repelleted, and resuspended in PBS. In different experiments, cells were maintained at 37°C, room temperature, or 10°C throughout the preparation. After an additional 15 min at the respective temperatures, cells were repelleted and resuspended in a microtubule stabilizing buffer (0.1 M 2-[N-morpholino]ethane sulfonic acid, pH 6.75, 1 mM MgSO₄, 2 mM EGTA, 0.1 mM EDTA, 0.1 mM GTP, 4 M glycerol, 1% Triton X-100). Soluble proteins were extracted at 37°C for 15 min. Residual cytoskeletal proteins were then solubilized in 0.1 M Tris, pH 6.8, 1% SDS. Protein concentrations were determined by bicinchoninic acid assay (Smith et al., 1985). The cytoskeletal fraction was boiled for 10 min to lower viscosity of the DNA. Both fractions were brought to identical volumes and equal fractions of each were then analyzed on SDS polyacrylamide gels followed by immunoblotting with either the cβ6- or cβ3-specific antisera. Serial dilutions (1.3-fold) of each sample were analyzed in adjacent slots of each blot. The autoradiographic image of each blot lane was quantified using a Zenieh Soft laser densitometer and a standard curve of integrated optical density versus amount of extract loaded was plotted. For each dilution (only points where both soluble and cytoskeletal values fell within the linear range of the analysis were utilized), the percentage of isotype in the cytoskeletal fraction was calculated as: percent cytoskeleton = 100 × (integrated OD of cytoskeletal fraction)/(sum of integrated OD of cytoskeletal and soluble fractions).

Results

Expression of the cβ6 Polypeptide in a Nonerythroid Cell

To determine whether the divergent cβ6 polypeptide would be recruited into and utilized in microtubules other than those of marginal bands in which it is found naturally, we constructed a hybrid gene (named pSV-cβ6) from which cβ6 could be expressed after transfection into a host cell. In the actual construct (shown in Fig. 1), the complete coding sequence from a cDNA clone of cβ6 (Murphy et al., 1987) was placed adjacent to the SV40 early promoter. At the 3' end of the cβ6 sequence, the SV40 T antigen polyadenylation site was included. In addition, to achieve as high a level of expression as possible, the plasmid also contained the complete SV40 origin of replication so it would be replicated when introduced into COS cells, a monkey cell line that constitutively synthesizes SV40 T antigen (Gluzman, 1981).

To test for expression of the cβ6 polypeptide after transfection, we utilized a rabbit polyclonal antibody that had been raised against cβ6 purified from chicken erythrocyte microtubules and that has been shown to bind specifically to the cβ6 polypeptide (Murphy et al., 1986). With this anti–cβ6 antibody, we used indirect immunofluorescence microscopy to analyze whether cβ6 synthesis and assembly into microtubules could be detected and localized after transfection of COS cells with pSV-cβ6. Fig. 2 displays microtubule arrays in the resultant cells as visualized on parallel coverslips stained with a tubulin monoclonal antibody (Fig. 2, A, C, and E) or the cβ6-specific antibody (Fig. 2, B, D, and F). It is readily apparent that while microtubules are promi-
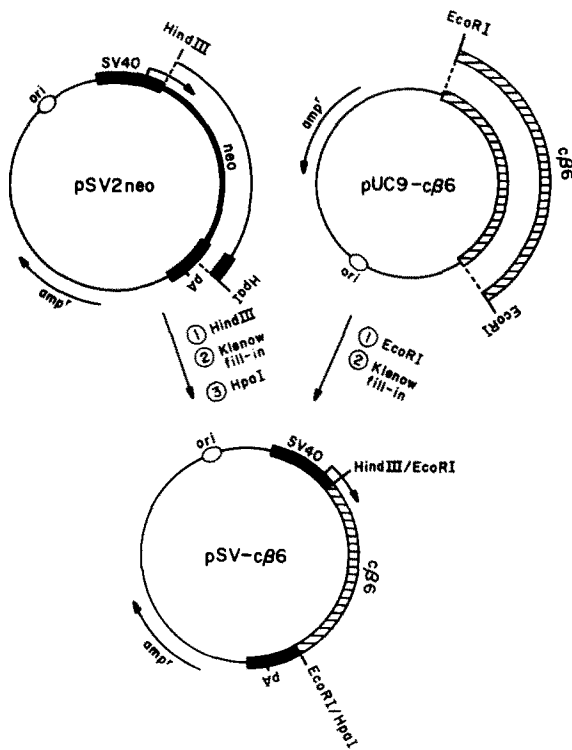
Figure 1. Construction of a hybrid gene for expression of the cβ6 polypeptide in animal cells. A cDNA copy of cβ6 mRNA beginning 70 bases 5' to the translation initiation site and terminating at the proximal site of polyadenylation (clone 8; Murphy et al., manuscript submitted for publication) was inserted in place of the neomycin resistance gene in plasmid pSV2-neo (Mulligan and Berg, 1980). The cβ6 cDNA insert (a subclone from an original λgtll clone) was excised from vector sequences with Eco RI, blunted by fill-in with Klenow polymerase and ligated to the pSV2 vector fragment by digestion of pSV2-neo at the Hind III site (which lies 3' to the transcription initiation site of the SV40 promoter) and at the Hpa I site (which lies just 5' to the SV40 polyadenylation signal). The final hybrid gene (pSV-cβ6) contained the SV40 DNA replication origin and early promoter, the cβ6 coding sequence, and the SV40 T antigen polyadenylation site.

nently stained in all cells by the monoclonal antibody (Fig. 2 *A*), only a small fraction (<3%) stained brightly with the cβ6 antibody (Fig. 2 *B*). (We presume that low transfection efficiency accounts for the small percentage of cβ6-positive cells.) However, in those few successfully transfected cells, it was clear that the cβ6 polypeptide was coassembled into an extensive array of cytoplasmic microtubules that was essentially indistinguishable from that identified by the monoclonal antibody either in low magnification images of whole cells (Fig. 2, *A* and *B*) or in higher magnifications (Fig. 2, *C* and *D*). The staining of individual fibers with either antibody appears uniform (Fig. 2, *C* and *D*).

Further, to determine whether cβ6 could also be incorporated into microtubules other than the interphase arrays, we looked for transfected cells that were in the mitotic phase of the cell cycle. Examples of mitotic spindles stained with the monoclonal anti-β-tubulin antibody and with the anti-cβ6 antibody are shown in Fig. 2, *E* and *F*, respectively. Clearly, there is no block to incorporation of the cβ6 polypeptide into

pole-to-chromosome, pole-to-pole, or astral microtubules of the spindle.

To confirm unambiguously the impression that in transfected cells cβ6 was found in most (and perhaps all) cytoplasmic and mitotic microtubules, a double-label immunofluorescence experiment was performed in which the same cells were stained both with the anti-cβ6 antibody and with an anti-β-tubulin monoclonal antibody. Fig. 3 displays the resultant micrographs in which cβ6 staining was visualized in the rhodamine channel (Fig. 3 *B*) and total microtubules were observed in fluorescein illumination (Fig. 3 *A*). In successfully transfected cells both antibodies identified an indistinguishable set of microtubule filaments, whereas in adjacent nontransfected cells only the monoclonal antibody showed staining (Fig. 3 *B*). (The very low level of staining observed in untransfected cells is probably due both to faint bleed through of fluorescein emission into the rhodamine channel or to nonspecific antibody binding, in that comparable staining is observed in mock transfected cells [not shown].) Although in cβ6-positive cells the possible existence of a small subset of microtubules that do not contain cβ6 cannot be eliminated by this kind of analysis, we have not detected any microtubules that are stained only by one of the two antibodies. We conclude that cβ6 must be coassembled into virtually all interphase microtubules.

A similar situation exists for mitotic microtubules in which spindles in transfected cells yield indistinguishable staining patterns in double-immunofluorescence experiments (data not shown).

In addition, because the fraction of cells expressing cβ6 was low (presumably the result of low transfection efficiency), we were able to examine whether transient expression of cβ6 in transfected cells prevented cell division after mitosis. To do this, 72 h after transfection we looked for cβ6 staining of presumptive daughter cells that had just emerged from cell division. Although the sensitivity of immunoblots with the anti-cβ6 antibody was not great enough for us to measure the relative levels of cβ6 and endogenous tubulins expressed in transfected cells, no evidence for inhibition of cell division as a consequence of cβ6 expression was apparent. Rather, in the midst of the >97% of cells not expressing cβ6, we were able to identify numerous pairs of cells that appeared to express high levels of cβ6 (see Fig. 3 *B*, for example). We presume that such pairs represent the daughter cells of a single transfectant that expressed cβ6 and subsequently divided successfully.

Coexpression of cβ6 and cβ3 Isotypes in Chicken Erythrocytes

While the demonstration that cβ6 was coassembled with other isoforms into interphase and mitotic microtubules of a transfected cell type that did not normally express it demonstrated that cβ6 was competent for coassembly in vivo with other tubulin isoforms, it remained unclear whether cβ6 was actually coassembled with other isotypes in its natural erythrocyte microtubules.

Our previous efforts (Sullivan and Cleveland, 1984; Sullivan et al., 1985, 1986*a*, *b*; Murphy et al., 1987) have demonstrated that β-tubulin is encoded in chicken by a family of seven genes that encode six different polypeptide isotypes. A summary of the genes, the encoded polypeptide isotypes
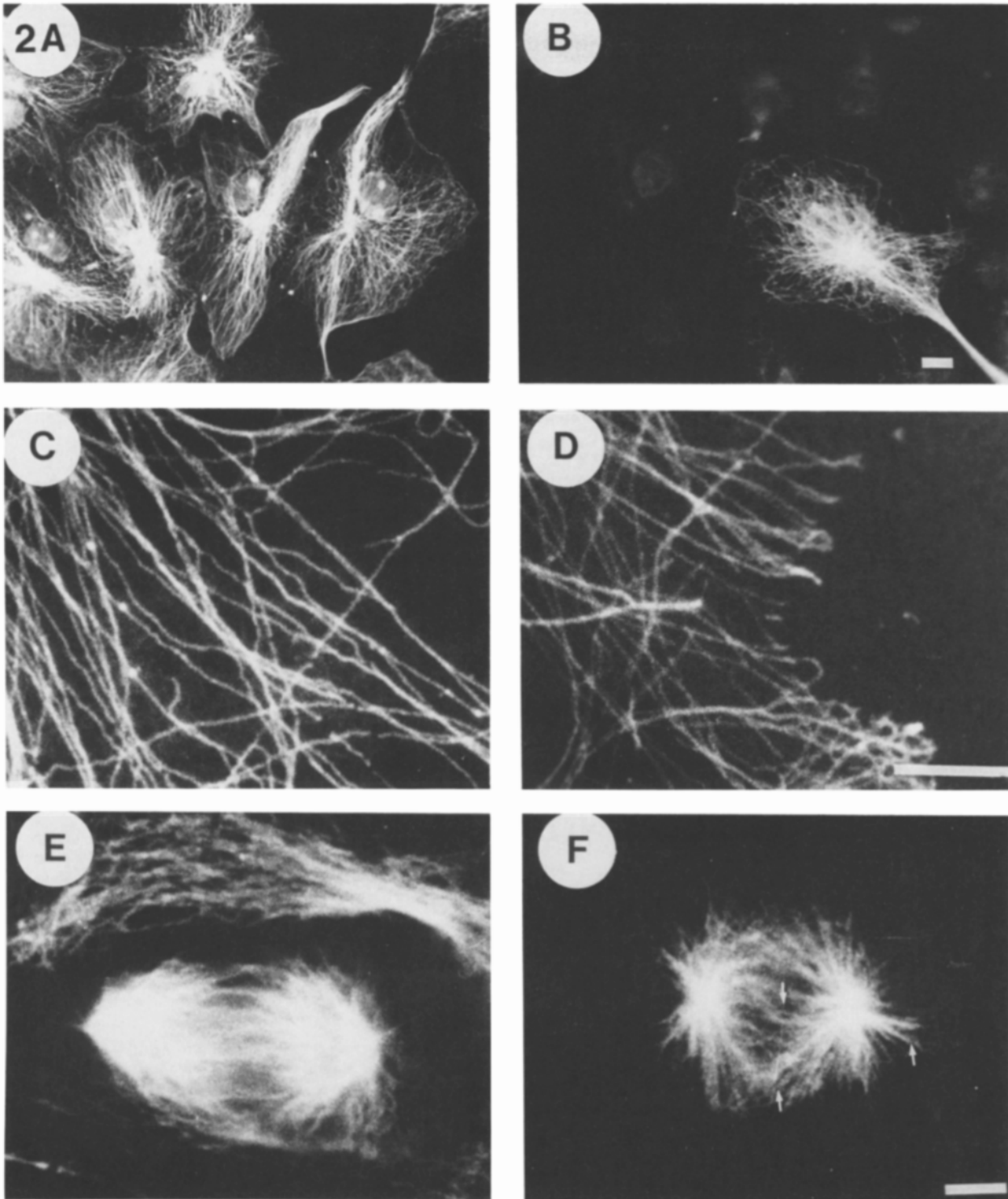
Figure 2. Indirect immunofluorescent visualization of microtubules in COS cells transfected with pSV-cβ6. (*A*) An epifluorescence micrograph of a field of COS cells that were mock-transfected and stained for all β-tubulins with a monoclonal antibody. Cytoskeletons were prepared by extraction of soluble proteins before fixation and staining. (*B*) Equivalent field of COS cells that were transfected with pSV-cβ6. Note the faint profile of the nuclei of untransfected cells surrounding the brightly fluorescent cells that are expressing cβ6. (*C* and *D*) Higher magnification views of single microtubules in the cytoskeletons of cells mock-transfected or transfected with pSV-cβ6, respectively. (*E*) Mitotic spindle of a COS cell stained with the monoclonal anti–β-tubulin antibody. (Image from the neighboring cells is out of focus due to lack of depth of field.) (*F*) Mitotic spindle in a cell transfected with pSV2-cβ6 and stained with the cβ6-specific primary antibody. Astral, polar, and kinetochore microtubules are all stained. Bars, 10 µM.
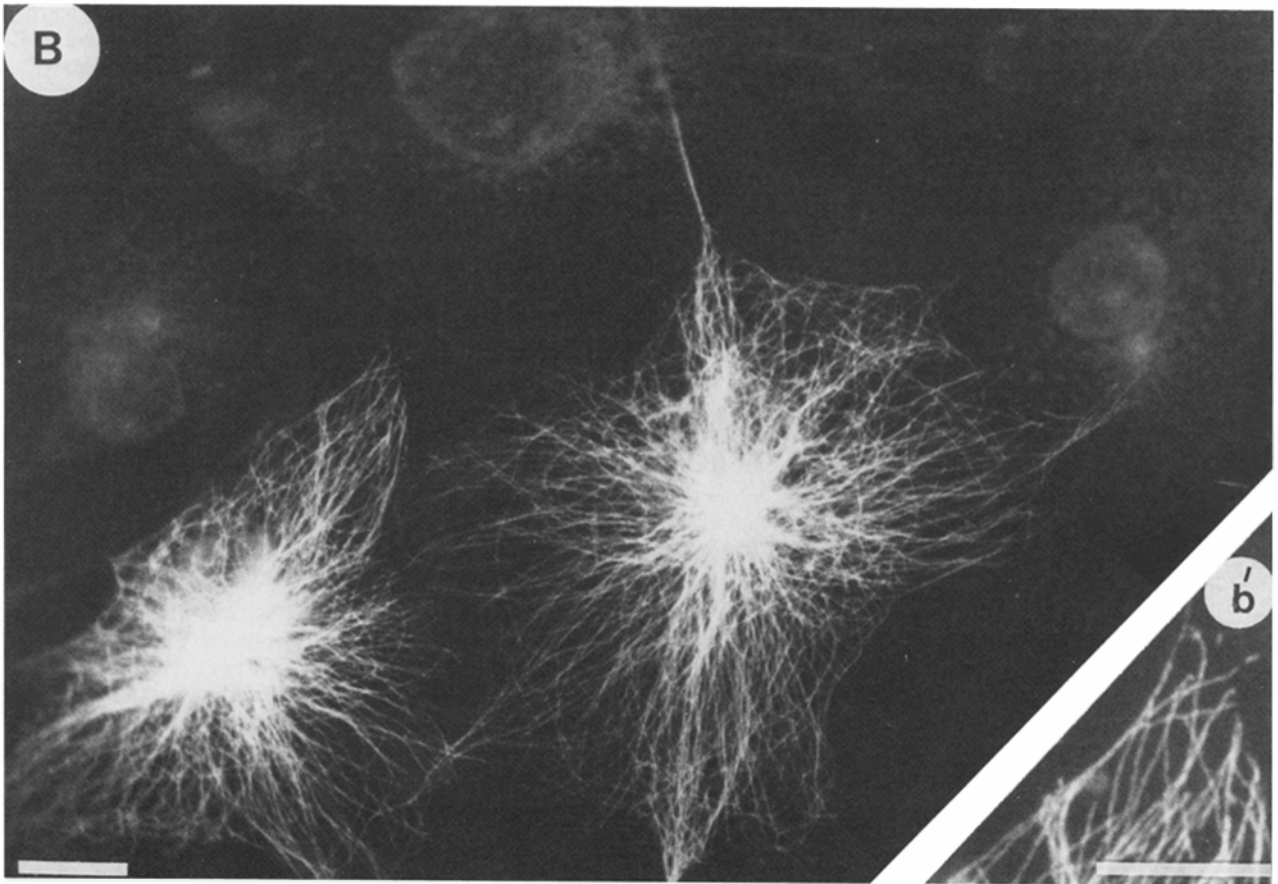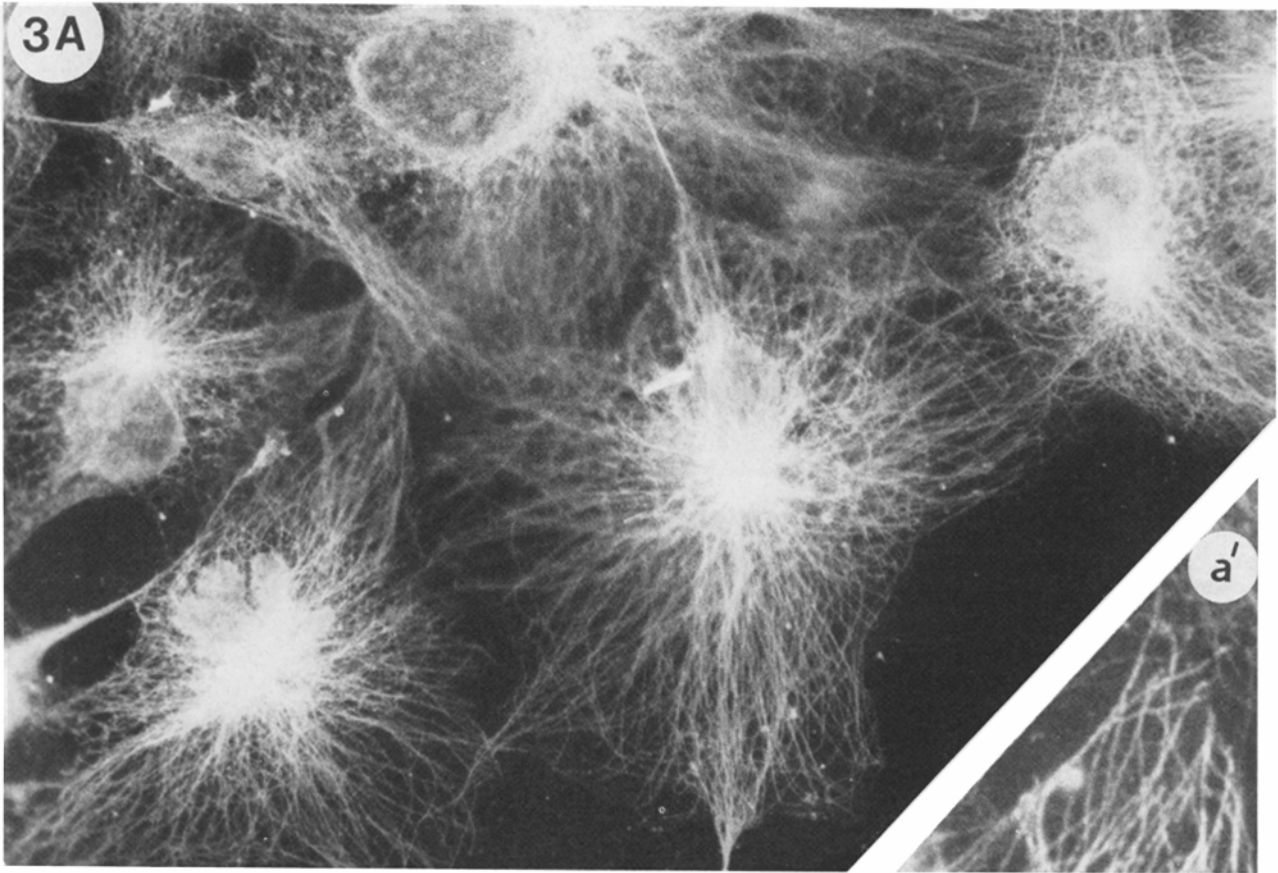
and the known patterns of expression is presented in Table I. (There are no data that suggest that additional β-tubulin genes or polypeptides are present in this organism.) In addition to the cβ6 polypeptides known to be expressed in erythroid cells (Murphy et al., 1986), we next determined which other β-tubulin isotypes are also found in red blood cells. To do this, we used synthetic peptide antigens corresponding to the extreme carboxy-terminal 9-17 amino acids to prepare antibodies that bind specifically to each of the remaining five β-tubulin isotypic classes (see Lopata and Cleveland, 1987). With the addition of the antibody to cβ6, these antibodies recognize the entire repertoire of β-tubulin isotypes known to be expressed in chicken. Using these antibodies, we examined proteins in an erythrocyte extract by protein blotting (Fig. 4 A). As is evident, only the anti–type c-IV antibody (that recognizes the product of the cβ3 gene) and the anti–cβ6 antibody bound to erythrocyte β-tubulins. By analyzing known amounts of cloned fusion proteins of both isotypes in adjacent slots of each immunoblot, we found that quantitatively β-tubulin in mature erythrocytes was composed of 95% cβ6 and 5% cβ3.

The immunoreactivity of the anti–cβ3 antibody (generated against a peptide corresponding to the carboxy-terminal nine amino acids of cβ3) with erythrocyte proteins is not due to cross-reaction with cβ6. Parallel immunoblotting of bacterial extracts containing cloned fusion proteins carrying the carboxy-terminal ~100 amino acid residues of cβ3 or cβ6 (Fig. 4 B) showed that anti–cβ3 or anti–cβ6 antibodies bound exclusively to cβ3 or cβ6 fusion proteins, respectively.

(That cβ3 is coexpressed with cβ6 is further supported by blot analysis of RNAs from erythroid cell population enriched in immature cells. Using cloned probes that specifically detect the RNAs from each of the seven chicken β-tubulin genes, only cβ3 RNAs and cβ7 RNAs are detected in addition to that for cβ6 (Murphy et al., 1987). Our inability here to detect cβ7 [type I] polypeptides in mature erythrocyte extracts may be due to the failure of type I to accumulate in erythrocytes; alternatively, the cβ7 RNAs detected on blots may actually be derived from the small proportion of non-erythroid cells that were present in the population of cells from which RNA had been prepared.)

Colocalization of cβ6 and cβ3 Tubulins in Marginal Band Microtubules

Examination of mature chicken erythrocytes by immunofluorescence with the anti–cβ6 antibody (Fig. 5 A) or with a monoclonal anti–β-tubulin antibody (Fig. 5 C) revealed intense staining of marginal band microtubules as previously reported (Murphy et al., 1986). Analysis using the anti–cβ3 antibody also revealed a very similar pattern of marginal band staining except that the intensity of staining varied markedly from cell to cell (Fig. 5 B). Specificity of the stain-
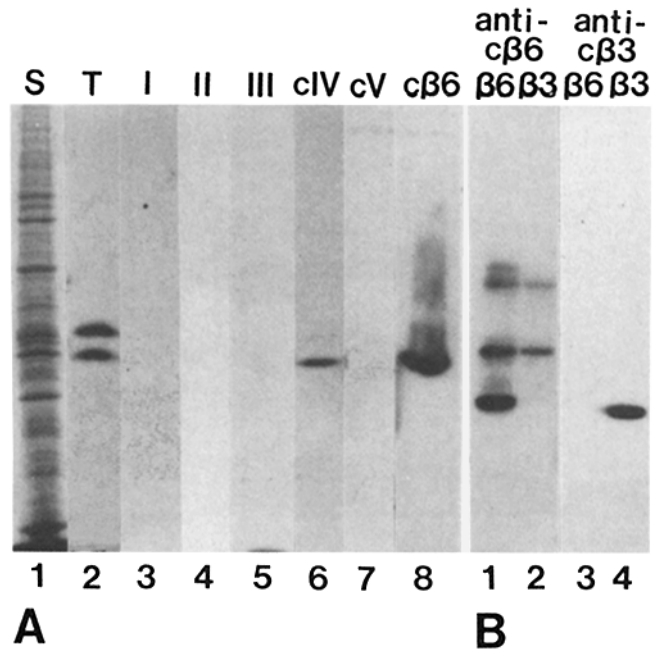

Figure 4. Analysis of β-tubulin isotypes expressed in chicken erythrocytes. (*A*) β-Tubulin isotypes in chicken erythrocytes. A whole-cell extract of mature chicken erythrocytes was electroblotted on parallel strips and the presence of each of six chicken β-tubulin isotypes probed with isotype-specific antisera followed by ^{125}I-protein A. Lane *1*, gel of erythrocyte extract stained with Coomassie Blue. Lane 2, immunoblot of erythrocyte extract using a polyclonal antibody that reacts with both α- and β-tubulins (Cleveland et al., 1980). Lanes 3–7, immunoblots using antibodies specific for type I, II, III, c-IV, and c-V: all isotype-specific sera were generated against synthetic peptide antigens (Lopata and Cleveland, 1987) corresponding to the extreme carboxy-terminal residues. Except for anti–type III for which whole serum was used, all other antipeptide antibodies were affinity-purified. (The polypeptide recognized at the bottom of lane *5* is also detected by preimmune serum.) Lane *8*, immunoblot with the rabbit polyclonal antibody of Murphy et al. (1986). (*B*) Specificity of the anti–cβ3 and anti–cβ6 antibodies. To demonstrate that the anti–cβ3 and anti–cβ6 antibodies do not cross-react, parallel protein blots of bacterial extracts containing cloned fusion proteins carrying the ~100 carboxy-terminal amino acids of (lanes *1* and *3*) cβ6 and (lanes *2* and *4*) cβ3 were analyzed with whole antisera to cβ6 (lanes *1* and *2*) or affinity-purified antisera to cβ3 (lanes *3* and *4*) and ^{125}I-protein A. (Note that although the anti–cβ6 antiserum binds to some bacterial proteins in both samples, it binds only to the cβ6 fusion protein and not to the cβ3 fusion.)

ing of anti–cβ3 antibody was verified by repeating the immunofluorescent protocols but in the presence of 3 µM cβ3 carboxy-terminal peptide. Anti–cβ3 antibody staining was completely abolished (Fig. 5 *E*), although addition of the

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Figure 3. Double-immunofluorescence micrographs of microtubules in COS cells transfected with pSV-cβ6. COS cells transfected with pSV-cβ6 were double stained with a monoclonal antibody to β-tubulin and with the cβ6-specific polyclonal antibody. A secondary goat anti–rabbit antibody conjugated to rhodamine was used to visualize anti–cβ6 staining. Monoclonal antibody binding was visualized with a goat anti–mouse secondary conjugated to fluorescein. (*A*) Monoclonal anti–β-tubulin staining visualized in fluorescein fluorescence; (*B*) same field of cells viewed in rhodamine fluorescence to display cβ6. (*Insets a' and b'*) Higher-magnification views of a portion of a single transfected cell visualized in fluorescein and rhodamine channels, respectively. Note that staining of individual fibers in both channels is indistinguishable. Bars, 10 µM.
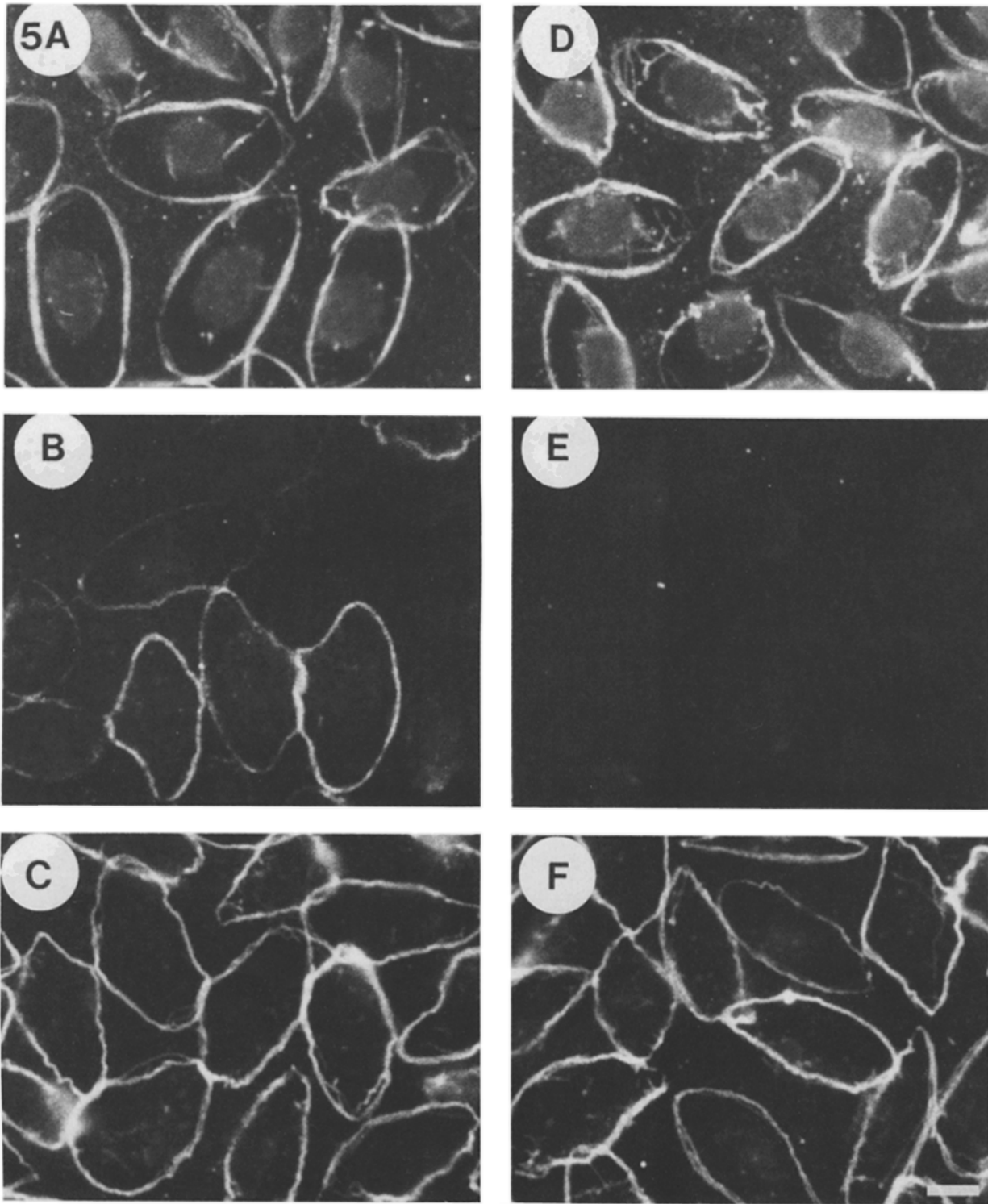
Figure 5. Indirect immunofluorescent visualization of cβ6 and cβ3 in marginal bands of chicken erythrocytes. Cells in *A, B,* and *C* were stained with a cβ6 isotype-specific polyclonal, a cβ3 isotype-specific polyclonal, and an anti–β-tubulin monoclonal primary antibody, respectively, and visualized with an appropriate secondary antibody conjugated with fluorescein. (*D, E,* and *F*) Micrographs from parallel coverslips stained as in *A, B,* and *C* except that each primary antibody was incubated with 3 μM of the cβ3 carboxy-terminal peptide (EEEAEEEAE) before staining cells. Note that preincubation of the anti–cβ3 antibody completely blocks marginal band staining in *E.* Bar, 5 μM.
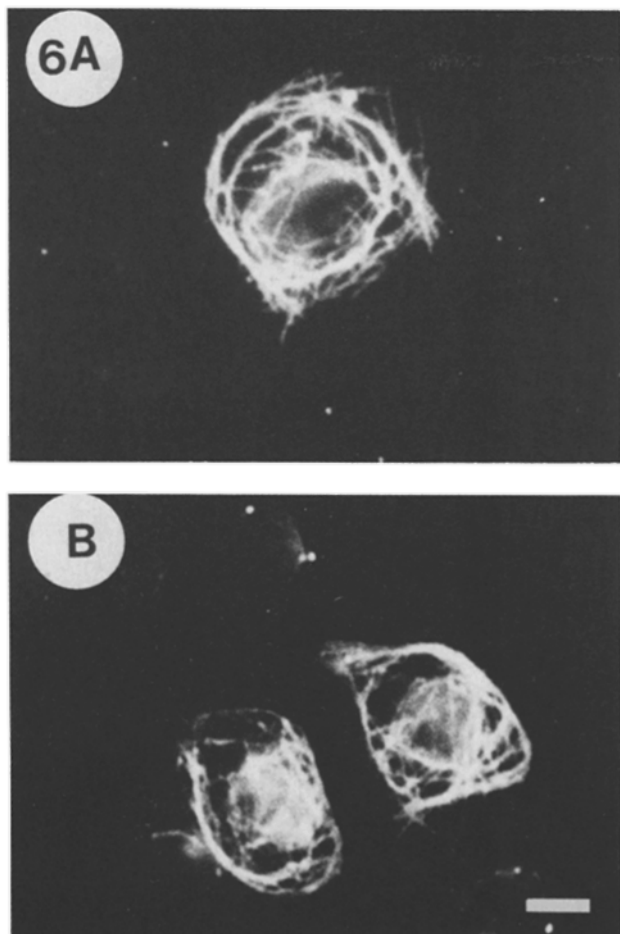
Figure 6. Indirect immunofluorescent staining of immature chicken erythrocytes with developing marginal bands. (*A*) Immature chicken erythrocytes were stained with the cβ6 isotype-specific primary antibody followed by a secondary antibody conjugated to fluorescein; (*B*) a parallel preparation of cells stained with the cβ3-specific primary antibody followed by a secondary antibody conjugated to fluorescein. Bar, 5 μM.

Figure 7. Quantitative analysis of soluble and polymeric cβ3 and cβ6 polypeptides in microtubules of mature erythrocytes. Erythrocyte proteins were fractionated into soluble and cytoskeletal fractions by lysis under conditions that are known to stabilize microtubules. Equivalent proportions of each fraction were analyzed by immunoblot analysis using the anti–cβ3 or anti–cβ6 antibodies. Samples in lanes *1–8* represent serial, 1.3-fold dilutions of the starting extracts. (*A*) Immunoblot of soluble (*M*) and cytoskeletal (*P*) fractions of erythrocytes prepared and extracted at 37°C. Blots were probed with anti–cβ3 and anti–cβ6 antibodies as indicated at the left. (*B*) Immunoblot of samples obtained from a erythrocytes prepared in parallel with the exception that prior to extraction the cells were maintained at 10°C.

same peptide to anti–cβ6 or monoclonal anti–β-tubulin antibody reactions had no effect on staining pattern or intensity (Fig. 5, *D* and *F*).

Colocalization of cβ6 and cβ3 Isotypes in Immature Erythrocytes

To test whether cβ3 and cβ6 were also coassembled in immature erythrocytes, we analyzed a population of cells enriched in developing erythrocytes (from the blood of an anemic chick). Immunofluorescence localization revealed that anti–cβ3 antibody identified a cytoplasmic array of developing marginal bands (Fig. 6 *B*) qualitatively indistinguishable from that seen with the anti–cβ6 antibody (Fig. 6 *A*). Unlike the mature cells, we did not observe differences in the amount of cβ3 from cell to cell, suggesting that the variability in mature cells might be related to age of the cell. (Because both primary antibodies were from rabbits, we were unable to perform a double-immunofluorescence experiment to localize both isotypes within a single cell.)

We conclude that the cβ3 and cβ6 isotypes are coassem-

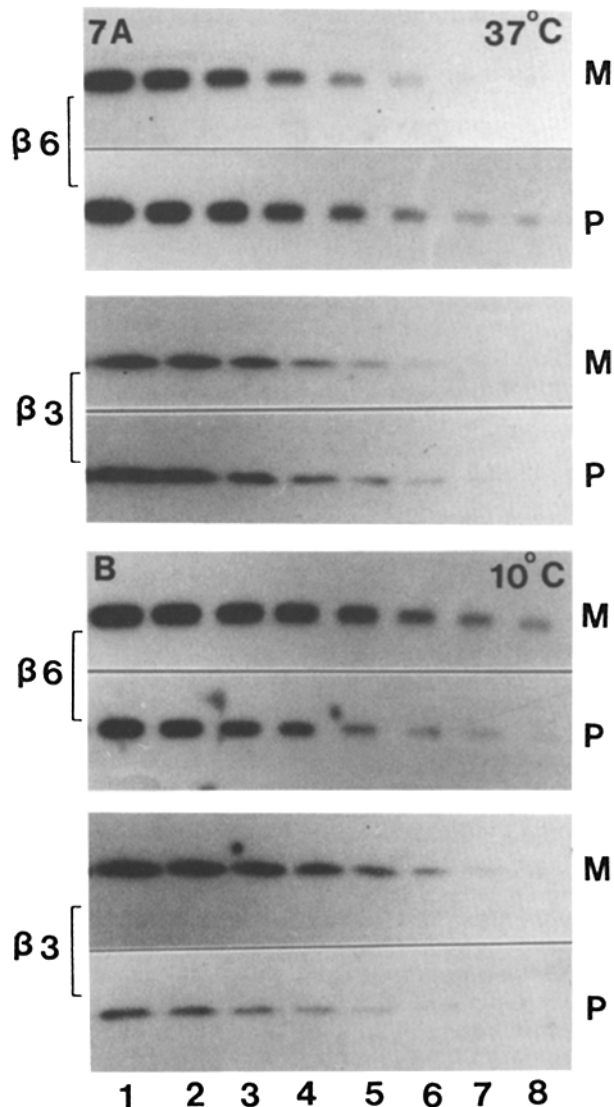bled into developing and mature marginal band microtubules.

Cold Lability of Microtubules Containing cβ3 and cβ6

The differential intensity of anti–cβ3 staining of adult red blood cells raised the intriguing possibility that, although cβ3 and cβ6 isotypes are coexpressed and (at the level allowed by light microscopy) colocalized in marginal band microtubules, the microtubules in mature cells that appear

to contain different apparent levels of cβ3 might possess some altered biochemical characteristic, such as stability to cold (Brinkley and Cartwright, 1975). To test this, we recovered red blood cells from whole blood of adult chickens, suspended the cells in PBS, and allowed them to attach to poly-lysine coated coverslips. Coverslips were then placed at 4, 10, or 20°C and 1, 5, 30 min, 2.5, and 4 h later samples were removed and analyzed by immunofluorescence for remaining microtubules (data not shown). While obvious diminution of both cβ6 and cβ3 signals was apparent in samples coded to 4 or 10°C, no differential stability of microtubules composed of either isotype was observable in any of the samples.

Ratios of Soluble and Polymeric cβ6 and cβ3 Tubulins in Erythrocytes

Although in using immunofluorescence we did not detect an overt difference in stability of cβ6- and cβ3-containing microtubules, we next determined whether cβ3 and cβ6 polypeptides were polymerized in vivo to the same extent, or whether one was preferentially utilized for microtubule assembly. To do this, we measured soluble and polymeric tubulin in erythrocytes from adult blood. Both fractions were analyzed by immunoblotting with anti-cβ6 and anti-cβ3 antibodies (Fig. 7). To insure that the immunoblotting was in the linear range of detection, 1.3-fold serial dilutions were analyzed in parallel and the resultant autoradiographs were quantified by densitometry. The quantified values for percentage of cβ3 and cβ6 in cytoskeletal compartments are presented in Table II. Inspection of the data revealed that when cells were maintained at 37°C throughout the preparation/extraction protocol, 57–70% of cβ3 and 52–64% of cβ6 was localized in the polymeric fraction. Although the precise fraction of subunits in polymer varied in two independent experiments, in both cases the levels of assembled cβ3 and cβ6 were comparable. However, a very different situation was encountered after incubation of the cells at 10°C for 30 min before extraction. Although microtubules containing both isotypes were disassembled by the cold incubation, quantitatively the proportion of cβ3 solubilized was almost twice that of cβ6. Further, this difference in stability of microtubules enriched in cβ3 was also observable after 40 min of incubation at room temperature prior to extraction. In this instance, less than half as much (22 ± 3%) of cβ3 was in the cytoskeletal fraction vs. (58 ± 7%) for cβ6 (Table II).

Thus, those experiments directly demonstrated that some in vivo microtubules enriched in cβ3 are less stable to temperature-induced disassembly than those enriched in cβ6.

Discussion

The discovery that animal genomes contain multiple functional β-tubulin genes that in turn encode a family of 6–7 β-tubulin polypeptides that differ in up to 21% of primary amino acid residues (Cleveland et al., 1980; Lopata et al., 1983; Hall et al., 1983; Sullivan and Cleveland, 1984; Farmer et al., 1984; Sullivan et al., 1985a, b; Lewis et al., 1985a, b; Wang et al., 1986; Sullivan and Cleveland, 1986; Murphy et al., 1987) has raised two general possibilities for the functional significance of this small gene family. Multiple genes might be utilized simply to provide alternative transcriptional promoter sequences for activation of tubulin expression during divergent pathways of cell differentiation (a possibility proposed most clearly by Raff [1984]). Alternatively, the polypeptide isotypes might themselves differ in some biochemical/assembly characteristic so as to confer some unique final property to an assembled microtubule.

Although it has been clear for some years that the first of these two possibilities is certainly true and that the repertoire of β-tubulins expressed in specific tissues and cell types is complex (e.g., Havercroft and Cleveland, 1984; Lewis et al., 1985; Wang et al., 1986), support for possible functional distinction among various isotypes has been limited to three lines of evidence. The first of these was discovery of phosphorylation of a specific isoform during neurite elongation (Edde et al., 1981; Gard and Kirschner, 1985). The second was identification of four classes of evolutionarily conserved isotypes of β-tubulin (Sullivan and Cleveland, 1986) that in the case of mammalian β-tubulins is nearly absolute (Wang et al., 1986). Third, differences in in vitro assembly properties of the cβ6 isoform (lower critical concentration and slower nucleation and elongation kinetics [Murphy and Wallis, 1986; Rothwell et al., 1986]) were identified.

However, whether isotypic sequence conservation is functionally important and whether the altered in vitro assembly differences are representative of in vivo differences has not been documented. To investigate this latter question, we have now analyzed the competence of the cβ6 polypeptide for assembly into microtubules in nonerythroid cells. To the limit of resolution available, the cβ6 polypeptide is incorporated into multifunctional classes of microtubules as a copolymer with other β-tubulin isotypes. These results support observations by Lewis et al. (1987) and by ourselves (Lopata and Cleveland, 1987) that in cells normally expressing multiple isotypes, microtubules are assembled as copolymers of available subunits.

We conclude from this that cβ6 is competent for assembly into divergent classes of microtubules and that cells do not

Table II. Quantitative Analysis of cβ3 and cβ6 Polypeptides in the Cytoskeletal Fraction*

Experiment no.	1	2	2	3
Temperature during cell preparation (°C)	37	37	10	20
cβ3 in cytoskeleton (%)	70 ± 5 (4)	57 ± 3 (5)	29 ± 2 (5)	22 ± 3 (5)
cβ6 in cytoskeleton (%)	64 ± 2 (10)	52 ± 2 (7)	40 ± 3 (5)	58 ± 7 (6)

* Points are shown ±SD. Number of independent determinations is shown in parentheses.

possess a mechanism with which the unusual cβ6 polypeptide can be excluded from some microtubule types. This outcome is not really surprising because (a) tubulins from all sources are known to coassemble in vitro, (b) cβ6 was known to be incorporated into erythroblast spindles (Murphy et al., 1986), and (c) a chimeric tubulin polypeptide consisting of 344 amino-terminal residues from chicken cβ2 tubulin linked to 113 carboxy-terminal residues from a yeast β-tubulin has previously been documented to assemble into all mouse fibroblast microtubules (Bond et al., 1986). Further, these results are in complete accord with recent experiments using a divergent mammalian β-tubulin isotype that in vivo is found exclusively in mouse erythroblasts and platelets. Those efforts (Lewis et al., 1987) documented that, upon transfection into HeLa cells, mβ1 tubulin is assembled into all classes of microtubules.

On the other hand, two observations of apparent, differential stability of microtubules enriched in the cβ6 isotype within its normal erythrocyte host provide support for an in vivo distinction between β-tubulin isoforms. The most convincing of these is our finding that microtubules containing higher proportions of cβ6 are more stable after incubation at low temperatures. Thus, cβ6 is biochemically distinguishable from cβ3 in vivo. A second observation is that the level of the cβ3 isotype in erythrocyte microtubules varies markedly from cell to cell, although no corresponding difference is observable for cβ6. Because it is known that the number of marginal band microtubules declines from an initial ∼20 in young cells to ∼5 in the oldest erythrocytes (Barrett and Dawson, 1974), this suggests that microtubules enriched in cβ3 polypeptides are preferentially lost from erythrocytes during cell aging. Although during aging (the half-life of a chicken erythrocyte is ∼30 d; Lucas and Jamroz, 1961) the loss of microtubules parallels the loss of other cellular components such as ribosomes (Barrett and Dawson, 1974), to our knowledge it is not known how microtubule loss affects erythrocyte function. However, considering the known distribution of the number of marginal band microtubules per cell in mature populations (a mean of 11 microtubules, with ∼90% of the cells with between 8 and 14; Miller and Solomon, 1984), if cβ3 polypeptides are lost from microtubules faster than cβ6 (as implied by the data summarized in Table II), then this could yield marked differences in the relative amount of cβ3 to cβ6 polypeptides between cells, a situation consistent with the immunofluorescence results of Fig. 5 B.

The preferential stabilization of cβ6 over cβ3 could derive from several potential sources. One possibility is the intrinsic greater stability of microtubules enriched in cβ6. Another possibility might be a preferential stabilization of cβ6 in microtubules as a consequence of binding to an as yet unidentified erythrocyte microtubule-associated protein or nucleation factor. Whatever the actual source, the greater stability of assembled cβ6 provides an in vivo example of a biochemically distinguishable tubulin isotype (although the physiological importance, if any, of this difference to marginal band microtubules or to erythrocyte function is not yet proven).

Consideration of all of the evidence demonstrates that, even though microtubules in all cells examined are copolymers of available isotypes (Lewis et al., 1987; Lopata and Cleveland, 1987), in at least one specialized cell type (chicken erythrocytes) isotypes can be distinguished in vivo. When coupled with previous evidence for the specific phosphorylation of a single β-tubulin isoform during neurite elongation (Edde et al., 1981; Gard and Kirschner, 1985), we conclude that at least within specialized microtubules isotype composition can specify (or reflect) some unique in vivo microtubule property.

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