Programmed Expression of β-Tubulin Genes during Development and Differentiation of the Chicken

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ABSTRACT We have previously demonstrated that the chicken genome contains at least four different, functional β-tubulin genes. By using gene specific probes we have now analyzed the relative levels of expression of the four encoded messenger RNA (mRNA) transcripts as a function of chicken development and differentiation. We have found that the RNA transcript from the β2 gene is present in large amounts in embryonic chick brain and is also preferentially expressed in spinal cord neurons, indicating that this transcript encodes the dominant neuronal β-tubulin polypeptide. The β3 mRNA is present in overwhelming amounts in RNA from chicken testis suggesting that this gene encodes a flagellar or meiotic spindle tubulin. However, both of these genes are transcribed to varying, but lesser, degrees in a number of additional cell and tissue types indicating that they are not neuronal or testis specific, respectively. β4′ transcripts are present at moderate levels in all cell and tissue types examined, suggesting that this mRNA encodes a constitutive β-tubulin polypeptide that is involved in an essential or housekeeping microtubule function. Transcripts from the β1 gene are a minor component of the β-tubulin mRNA populations in all cells and tissues tested. Overall, we conclude that specific β-tubulin mRNA species are expressed in markedly different ratios in different tissues in the chicken. Such developmental regulation may reflect the function(s) of the individual β-tubulin polypeptides or, alternatively, may be required for precise control of tubulin gene expression in cells that utilize microtubules for divergent purposes.

Microtubules, composed principally of heterodimers of one α- and one β-tubulin subunit, are filamentous polymers utilized in a number of diverse cellular processes. They are the major structural components of eucaryotic cilia and flagella and of mitotic and meiotic spindles. Microtubules also function in maintaining intracellular cytoplasmic structure and in several aspects of intracellular transport.

Many groups have reported microheterogeneity in both the α- and β-tubulin polypeptides as judged by one- or two-dimensional PAGE or by high-resolution isoelectric focusing (1–3). However, the nature of the differences in the tubulin subunits, whether due to posttranslational modification of one gene product or to differences in the polypeptide products of multiple genes, has only recently become approachable. In this regard, molecular cloning techniques have demonstrated that, in all but the simplest eucaryotes (yeast), there are multiple genomic sequences for both α- and β-tubulin, although the exact number and arrangement appears to vary widely (see reference 5 for a review).

In the best-studied invertebrate system, *Drosophila*, four α- and four β-tubulin genes have been identified. All four α- (6) and four β- (7) tubulin genes have been shown to be functional and to be expressed in a complex pattern during development. Moreover, by analysis of tubulin mutations that cause male sterility, one β-tubulin gene has been shown to be expressed only in testis (8). A mutation at this locus causes defects in meiosis followed by abnormal nuclear shaping and axoneme assembly. This has been interpreted to mean that this β-tubulin subunit is involved in the formation of microtubules utilized in all of the above processes.

In vertebrate genomes, however, the tubulin sequence complexity is more difficult to dissect. Mammalian genomes typically contain 15–20 sequences homologous to α-tubulin and a further 15–20 sequences homologous to β-tubulin (9). In the human tubulin gene family, Cowan and co-workers (10–12) have shown that, of the 10 β-tubulin genomic sequences so far examined, only three are authentic genes, whereas the majority are pseudogenes that have a variety of

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lesions that prevent their translation into a functional polypeptide. It is not yet known how many more of the remaining β genes are truly functional.

However, in the chicken genome we initially identified 4–5 sequences with strong homology to a cloned chick brain α-tubulin complementary DNA (cDNA) and another 4–5 sequences with strong homology to a cloned chick brain β-tubulin cDNA (9). Previously, we cloned and characterized the four gene segments, which have detectable sequence homology to both the *N*-terminal and *C*-terminal regions of a chick β-tubulin cDNA clone (13). With the exception of two highly divergent segments that we have recently identified (Havercroft, J. C., D. B. Murphy, and D. W. Cleveland, unpublished observations) these four genes appear to comprise the entire set of β-tubulin sequences in the chicken genome that are detectable by homology to existing cloned β-tubulin sequences. Moreover, we have shown the presence in chicken cells of four distinct stable messenger RNAs (mRNAs) each derived from a different β-tubulin gene. Although we initially identified one of these mRNA transcripts to each of the four cloned genes (13), we now show that the assignment of one of the transcripts is surprisingly more complicated than originally anticipated. Nonetheless, by using hybridization probes constructed from each of the four cloned β-tubulin genes, it is now possible to begin to investigate whether differential tubulin gene expression contributes in an important manner to the assembly and function of different kinds of microtubules within a single cell or tissue type.

MATERIALS AND METHODS

Cells and Tissues: Brain and total embryo RNAs were isolated from embryos of the inbred chicken line #003. These embryos were kindly provided by Dr. Hans Abplanalp (University of California, Davis). Tissues for the isolation of RNA were taken from adult birds in all cases. Hepatocytes were prepared by collagenase digestion of the liver of a 16-d-old chicken followed by plating for 4 h. The procedure used minimizes the number of Kupffer cells and red blood cells (14). Chicken fibroblasts were isolated from 10-d embryos by trypsinization and RNA was isolated after two subculturings. Neurons were isolated from the spinal cord ganglia of a 6-d chick embryo and maintained for 6 d in culture. Glial cells were isolated by trypsinization from the cerebral hemispheres of a 19-d embryo. They were then subcultured three times in an 8-d period at which time no neurons could be detected visually. Smooth muscle cells were isolated from the gizzard of an 11-d chick embryo and maintained in culture for 2 d. The cultured cell population consisted of ~85–90% muscle cells, as judged by immunofluorescent staining with an antibody to desmin. For striated muscle cells, developing myoblasts were isolated from the breast muscle of an 11-d embryo. They were plated in control medium for 3 d followed by addition of cytosine arabinoside for 48 h to kill dividing cells and then grown in normal medium for an additional 24 h. By this stage cell fusion had occurred forming multinucleated myotubes. Secondary chondroblasts were isolated from 11–12-d chick embryo vertebral columns and then plated for 10 d before RNA isolation. Red blood cells of the primitive line were collected from 5-d embryos and were free of white blood cells inasmuch as the latter are not present in chicken at this stage of development.

Preparation of RNA: Total RNA was prepared using the CsCl step method of Chirgwin et al. (15). Tissues or whole embryos were frozen in liquid nitrogen then disrupted in guanidine isothiocyanate using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). RNA was prepared from plated cell cultures by lysis with guanidine isothiocyanate followed by homogenization. Poly A-containing RNA was selected by chromatography on oligo-(dT) cellulose (type T3, Collaborative Research Inc., Waltham, MA).

Gel Electrophoresis and Blotting: Two-dimensional PAGE of proteins was according to O'Farrell (16). Gel electrophoresis and blotting of RNA and DNA was as previously described (13). Denaturing RNA gels were cast from 0.8% agarose containing 2.2 M formaldehyde (17). Quantitation of signals of ^{32}P-labeled probes hybridized to RNA immobilized on nitrocellulose filters ("Northern blots") was achieved by densitometry of autoradiograms using a Kontes densitometer (Kontes Co., Vineland, NJ) equipped with a numerical integrator (Hewlett-Packard, Palo Alto, CA). To determine the linearity of the densitometer/film response, a series of known quantities of ^{32}P end-labeled, Hind III-cut, lambda phage DNA was run on an agarose gel, dried onto DEAE paper, and exposed to film (at −80°C with DuPont Lightning Plus intensifying screens [DuPont Instruments, Wilmington, DE]) for times varying from 5 h to 5 d. The resultant autoradiograms were scanned using the densitometer and the integrated densities corresponding to each band were plotted against the known number of counts loaded. From these data the linear range of the densitometer–film response was determined, and all subsequent autoradiographic exposures were maintained within this range.

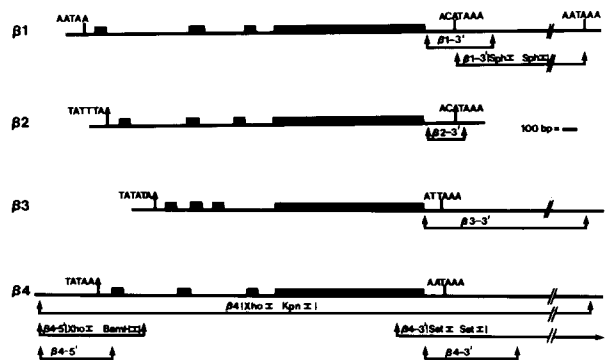

FIGURE 1 Gene-specific probes constructed from the β1, β2, β3, and β4 genes. A schematic drawing of each of the four β-tubulin genes β1, β2, β3, and β4 is shown. The thick lines denote protein coding sequence regions and the thin lines denote intron or flanking DNA sequences. The positions of the putative promotor or TATA box and consensus polyadenylation signal are shown for each gene. The portions of each gene contained on each gene specific probe (see Materials and Methods) are also illustrated.

Preparation of ^{32}P-labeled Hybridization Probes: Purified, cloned DNA segments specific for actin, α-tubulin, each individual β-tubulin gene, or ribosomal RNA were labeled with ^{32}P by the random priming method of Shank et al. (18), as described previously (13). All probes were labeled to approximately equal specific activity with the exception of the β2-3′ probe which gave consistently four- to fivefold lower incorporation. For detection of actin mRNAs, the cloned cDNA from pA1 (19), which contains the entire coding sequence for chicken β-actin, was labeled as a probe. α-Tubulin mRNAs were detected with the cloned cDNA from plasmid pT1, which contains 92% of the protein-coding region for a chick brain α-tubulin (20). Total β-tubulin mRNAs were detected with the cloned cDNA from pT2 (20), which contains the complete coding region of a chick brain β-tubulin mRNA. In fact, the cDNA of pT2 represents a cloned copy of the mRNA encoded by chick gene β2 (13).

Specific probes for each cloned chicken β tubulin gene were isolated as detailed below. For ease of reference, the region of each gene contained on each probe is diagrammed in Fig. 1. (For restriction maps of each gene see Lopata et al. [13]).

β1-3′. For gene β1, a 475-base pair (bp)[1] fragment was used. This fragment extends from the Xmn I site 24 bases 3′ to the end of the β1-coding region and terminates at the distal Bst E II site.

β1-3′(SphI-Sph I). For gene β1, a second 3′ gene-specific probe was also constructed. This probe extends from the Sph I site 217 bp 3′ to the translation termination codon through to the second Sph I site 2 kilobase pairs (kb) downstream.

β2-3′. For gene β2, a 240-bp gene-specific probe corresponding to the 3′ untranslated region of the gene was isolated from the cDNA clone pT2. The actual probe starts at the Dde I site 30 bases 3′ to the translation termination codon and extends through to the poly A addition site.

β3-3′. For gene β3, a 2-kb fragment was constructed by Bal 31 double-stranded exonuclease digestion of the cloned β3 gene and the fragment was subcloned into M13 mp9. This final subclone begins at the T of the original TAA translation termination codon and extends to the downstream Xho I site. Consequently, it contains solely a 3′ noncoding region sequence as well as some flanking genomic DNA.

β4(XhoI-KpnI). For gene β4, an initial probe utilized extended from the Xho I site ~480 bp 5′ to the translation initiation codon through to the Kpn I site 2.5 kb 3′ to the translation termination codon. This probe thus contains the entire coding region as well as the 5′ and 3′ noncoding regions.

[1] *Abbreviations used in this paper:* bp, base pair; kb, kilobase pair.

β4-5′(XhoI-Bam HI). For gene β4, one 5′ gene-specific probe that was utilized extends from the same 5′ Xho I site (see above) through the first exon (57 bases of coding sequence) and terminates at the Bam HI site in the first intron.

β4-5′. For gene β4, a 5′ specific probe that contains no coding sequence was also constructed using Bal 31 exonuclease. This probe again extends from the 5′ Xho I site to within 1 bp of the translation initiation codon.

β4-3′. For gene β4, a 3′ specific probe carrying sequences adjacent to the translation termination codon was obtained. This probe begins at the Nar I site 7 bases 5′ to the translation termination codon and extends to the downstream Pst I site. It thus contains 7 bp of coding and 652 bp of flanking noncoding sequence.

β4-3′(SstI-SstI). For gene β4, a second 3′ probe spanning a larger 3′ region was also utilized. This probe was isolated from the original β4 gene, subcloned into pBR322 at the Sph I site (13). This probe begins at the SstI site in the 3′ end of β4 and extends through pBR322 to the SstI site in the far 5′ flanking region of this gene. It contains 217 bp of *C*-terminal coding sequence, 2.6 kb of 3′ flanking sequence, and ~300 bp of 5′ flanking sequence.

Ribosomal RNA. A probe for ribosomal RNAs was prepared from pX1r101, a plasmid that contains the *Xenopus laevis* 18S and 28S ribosomal sequences. These *Xenopus* rDNA sequences cross-hybridize efficiently to both 18S and 28S rRNA species in the chicken.

RESULTS

Construction and Characterization of DNA Probes Specific for Each Chicken β-Tubulin Gene

Previously we demonstrated that the mRNA species from the four chicken β-tubulin genes could be distinguished either by size or by the use of hybridization probes derived from the 3′ ends of the corresponding genes. By using the latter approach, the β1 gene was shown to preferentially hybridize to an mRNA ~4,000 bases in length, whereas the β4 gene preferentially hybridized to an RNA varying in size between 3,500 and 3,700 bases (13). This heterogeneity in transcript size (which we originally reported—see Lopata et al. [13]) is most likely due to allelic differences in the progenitor gene in different chicken populations because we have not observed it in RNA samples derived from individual chickens or in RNA prepared from a line of inbred chickens where a single predictable mRNA species is produced (data not shown). Although our previous probes very strongly suggested that the transcripts detected represented the RNAs corresponding to the β1 and β4 genes, we have now utilized partial DNA sequence data to construct more definitive probes for both β1 and β4 (see Materials and Methods and Fig. 1 for details). The new β1-3′ probe, which begins 24 bp 3′ to the stop codon for translation and extends 475 bp downstream, confirms the identification of the 4,000-base transcript as a product of the β1 gene (Fig. 2, lane *2*). In addition, it identifies an 1,800-base transcript also derived from this gene (Fig. 2, lane *2*). It is likely that the 1,800-base transcript is produced using the polyadenylation signal (ACATAAA) located 210 bp 3′ to the TGA translation termination signal (Sullivan K. F., and D. W. Cleveland, unpublished observations). The 4,000-base mRNA species is probably produced by readthrough of this polyadenylation signal because the β1-3′(SphI-SphI) probe, which extends an additional 2kb downstream, strongly recognizes this larger mRNA (Fig. 2, lane *3*). Neither of these probes hybridizes detectably to any other chicken β-tubulin gene (data not shown).

A 3,500-base β-tubulin mRNA (easily seen in mRNA from chondroblasts with a β-tubulin coding sequence probe—see Fig. 2, lane *1*) was originally identified to be the product of the β4 gene by virtue of its overwhelmingly preferential hybridization to the β4(XhoI-KpnI) probe, which contains the complete β4 coding sequence in addition to both 5′ and 3′

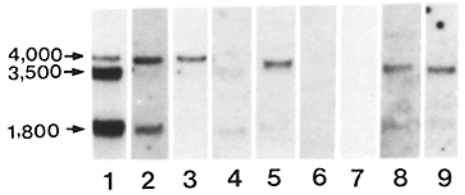

FIGURE 2 Identification of the RNAs encoded by the β1 and β4 genes. Chondroblast (lanes *1–3*) or smooth muscle (lanes *4–9*) poly-(A) containing RNA was electrophoresed on denaturing agarose gels, transferred to nitrocellulose, and hybridized to the following probes: (*1*) pT2, the β-tubulin cDNA; (*2*) β1-3′; (*3*) β1-3′ (SphI-SphI); (*4*) pT2; (*5*) β4(XhoI-KpnI); (*6*) β4-3′; (*7*) β4-5′; (*8*) β4-5′(XhoI-BamHI); (*9*) β4-3′(SstI-SstI).

flanking regions (Fig. 2, lane *5*). To confirm this identification, we constructed the β4-3′ probe, which contains only 7 bp of coding and extends 652 bp into the 3′ flanking region. To our surprise, we have been unable to detect hybridization of this probe to the 3,500-base β-tubulin RNA or to any other chicken RNA species by Northern blot analysis (Fig. 2, lane *6*). Furthermore, a probe (β4-5′) constructed to contain only a 5′ noncoding sequence also fails to identify any RNA species (Fig. 2, lane *7*). Probes that include small portions of *N*-terminal [β4-5′(XhoI-BamHI)] or *C*-terminal [β4-3′(SstI-SstI] coding regions (57 and 217 bp, respectively) from β4, however, hybridize strongly and preferentially to the 3,500-base mRNA (Fig. 2, lanes *8* and *9*). Such preferential hybridization to the 3,500 base mRNA is not seen with coding sequence probes constructed from β1, β2, or β3 (see, for example, lane *4*, which has been probed with the coding region of β2). We, therefore, are left with two alternative explanations for the source of this 3,500-base β-tubulin mRNA and its relationship to the cloned β4 gene: first, it remains possible that the β4 gene that we have isolated is the progenitor of this 3,500 base species but that the immediate 5′ and 3′ flanking regions are spliced out of the mature mRNA; second, it is possible that the 3,500-base transcript derives from a previously unidentified β-tubulin gene, which has very strong coding region homology to the β4 gene, but differs in its 5′ and 3′ flanking regions. Although we cannot, at present, unambiguously distinguish between these two alternatives, we would hasten to emphasize that, regardless of whether β4 or its putative sister gene actually gives rise to the 3,500-base RNA (which we shall designate β4′), this transcript has been shown to be a functional β-tubulin mRNA (13) and can be unambiguously identified by its size and preferential hybridization to β4 coding-region probes.

The mRNA species from the β2 and β3 genes are both ~1,800 bases in length and co-migrate on denaturing agarose gels with the 1,800-base transcript from the β1 gene. Hence, to distinguish these transcripts, it was again necessary to construct gene-specific probes. For β2, we constructed a subclone of the plasmid pT2, which contains a cDNA copy of the mRNA encoded by the β2 gene (13). From the known sequence of pT2 (20), we isolated a 240-bp fragment, which begins 30 bases 3′ to the translation termination codon and continues on through to the poly A addition site (see Fig. 1). This fragment hybridizes only to the β2 gene and is designated β2-3′. For the β3 gene, a 2-kb fragment (to be referred to as β3-3′) was constructed by Bal 31 exonuclease digestion of the β3 genomic clone. By nucleotide sequence analysis (Sullivan K. F., and D. W. Cleveland, unpublished observations) we have shown that this particular subclone begins at the T of

the TAA stop codon for translation, extends through the 3′ untranslated region of the β3 gene, and continues on into the flanking DNA sequences (see Fig. 1).

The explicit assumption in the isolation of each of these 3′ transcribed, untranslated regions is that they are not conserved and that there will be no cross-hybridization between them. To test this, we took the β1, β2, β3, and β4 genes (individually subcloned into pBR322) and cut each with appropriate restriction enzymes to generate fragments of 4–5 kb in length, one of which would contain the entire coding and 3′ untranslated region for that gene. These were electrophoresed as five identical sets on agarose gels, transferred to nitrocellulose, and probed with either ^{32}P-labeled pT2, the coding sequence containing β-tubulin cDNA clone or one of the four 3′ gene-specific probes (β1-3′, β2-3′, β3-3′, or β4-3′). As expected, pT2 hybridizes to the coding region of each of the four genes, but the gene-specific probes recognize their parent genes only. This was true even after very long exposure. The specificity of the probes was further confirmed by hybridization to Eco R1-digested chicken genomic DNA. Each probe recognized only the appropriate sized fragment corresponding to its parent gene.

Determination of the Program of Expression of Each β-Tubulin Gene during Chick Embryo Development

After having constructed appropriate probes with which we could distinguish each β-tubulin mRNA transcript, we sought to determine the program of expression of each β-tubulin gene. Initially, we wished to test whether β-tubulin genes are differentially expressed as a function of chick embryo development. Total RNA was isolated from whole embryos that had been incubated for times from 3 to 12 d after fertilization and equal amounts of RNA were electrophoresed on denaturing agarose gels. Identical gels were transferred to nitrocellulose and hybridized to the β-tubulin cDNA (Fig. 3*a*), the β2-3′ gene-specific probe (Fig. 3*b*), the β3-3′ gene-specific probe (Fig. 3*c*), the α-tubulin cDNA probe (Fig. 3*d*) or the β-actin cDNA probe (Fig. 3*e*). Subsequently, to control quantitatively for any differences in apparent abundance that might result from unequal loading or blotting of RNA aliquots, all blots were rehybridized to pX1r101, a recombinant plasmid that recognizes both 18S and 28S ribosomal transcripts. As is evident in Fig. 3, there are large differences in the levels of expression of the four genes. β2 (Fig. 3, *a* and *b*) is by far the most abundant species, whereas β3 (Fig. 3, *a* and *c*) is barely detectable. This is true despite the four- to fivefold lower specific activity of the β2-3′ probe as compared with all of the other probes utilized (see Materials and Methods). β4′ is present at intermediate levels as determined by hybridization to β2 (Fig. 2*a*) or β4 (data not shown) coding region probes. β1, as judged by the level of the 4,000-base transcript, is in low abundance (Fig. 3*a*). The β1-3′ gene-specific probe (data not shown) indicates that there is an even lower level of the 1,800-base transcript from β1 (even after correcting for the differing number of bases in the two transcripts which are homologous to the probe).

The relative level of each RNA transcript at each stage of development of the embryo was determined by densitometry of the autoradiograms of duplicate experiments. The resulting values were then corrected for variations in the amount of 28S rRNA and the results plotted in Fig. 4*a* for β1–β4′,

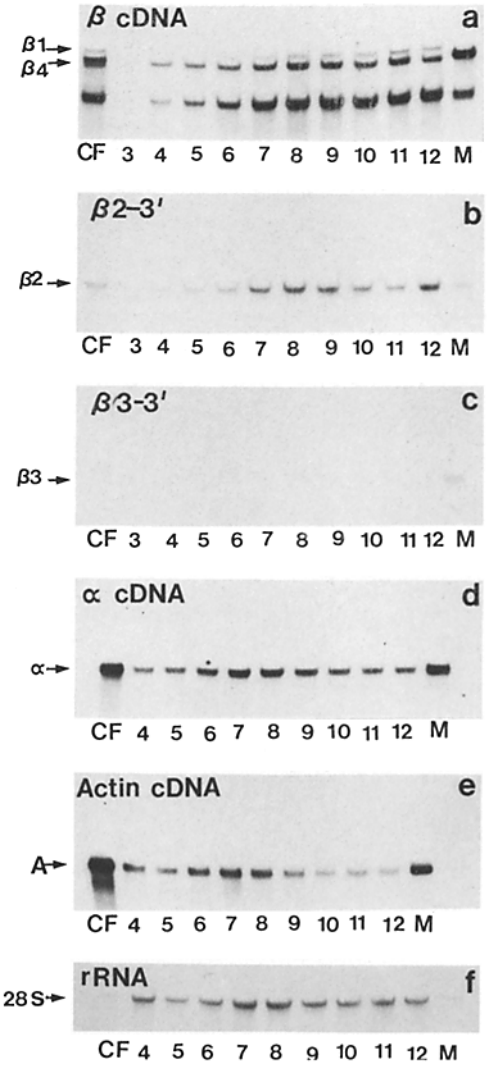

FIGURE 3 Differential expression of tubulin and actin mRNAs during chick embryo development. Total RNA was isolated from whole chick embryos of 3–12 d of development. Equal aliquots of each sample (12 μg) were electrophoresed on five identical denaturing agarose gels, transferred to nitrocellulose, and hybridized to the following probes: (*a*) pT2, β-tubulin cDNA; (*b*) β2-3′ gene specific probe; (*c*) β3-3′ gene specific probe; (*d*) pT1, α-tubulin cDNA; (*e*) pA1, β-actin cDNA; (*f*) 28S ribosomal RNA probe. Tracks: *CF*, secondary chick fibroblasts; *M*, MSB, a cloned chick T lymphocyte cell line; *3–12*, RNA from 3–12-d-old embryos.

respectively. In addition, α-tubulin and actin RNA levels were also quantitated in a similar manner and plotted in the bottom two panels in Fig. 4*a*. It can be seen that the 4,000-base transcript from β1 rises from an undetectable level in embryos younger than 7 d to a low level by the 12-d stage. The corresponding plot for the 1,800-base transcript is very similar (data not shown). On the other hand, β2 rises 16-fold from day 4 until day 9 and then falls once more to its initial low level. β3 remains essentially constant but in low abundance throughout. Both β4′ and α-tubulin rise along with β2, but less dramatically, showing a threefold increase from day 4 until day 8. From this point they decline in amount. In contrast (Fig. 4*a*, *bottom*), the actin RNA level peaks much earlier (at 6 d) after which point it then slowly declines in amount.

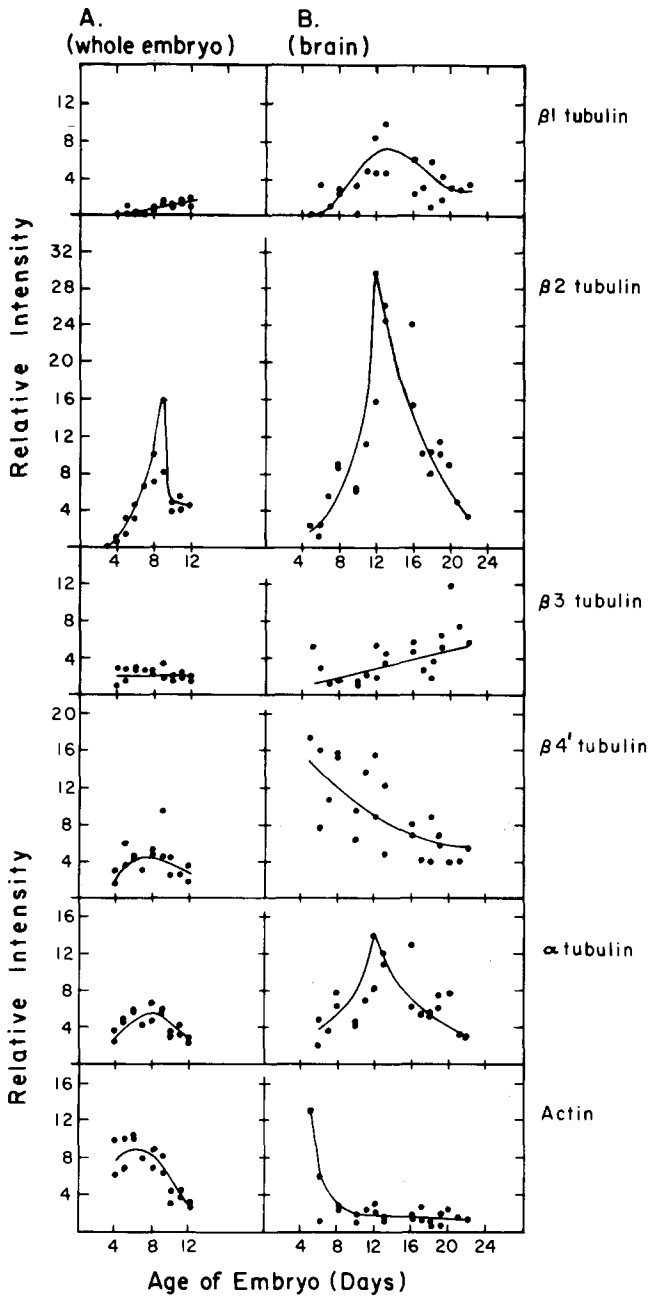

FIGURE 4 Quantitation of β-tubulin mRNAs in chick embryo and embryonic brain during development. The autoradiograms displayed in Figs. 2 and 4 were scanned using a densitometer, and the areas under the bands were calculated by using an integrator. The autoradiograms from duplicate gels were quantitated in the same way and all the results corrected for variations in the level of total RNA as judged by the 28S ribosomal RNA species. The data were plotted as the relative intensity of the band as calculated by the integrator versus the age of the embryo. (*A*) RNA levels in the embryo; (*B*) RNA levels in the brain.

Determination of the Program of Expression of β-Tubulins during Chick Brain Development

As chick brain is frequently used as a source of tubulin and has been shown by high resolution isoelectric focusing (3) and two-dimensional PAGE (1) to be heterogeneous in its tubulin content, we have also examined whether there are changes in the levels of the mRNAs from the different β-tubulin genes during embryonic brain development. To do this we isolated total RNA from chick embryo brains from 5 d of incubation until hatching (22 d). Equal amounts of RNA were again electrophoresed on denaturing agarose gels, transferred to nitrocellulose, and then probed with the β-tubulin cDNA (Fig. 5*a*), the β2-3′ gene-specific probe (Fig. 5*b*), the β3-3′ gene-specific probe (Fig. 5*c*), the α-tubulin cDNA (Fig. 5*d*) or the β-actin cDNA (Fig. 5*e*). As in the whole embryo, β2 is the dominant transcript although it is even more enriched in brain tissue. β4′ is expressed at moderate levels, with β1 present at lower levels and β3 barely detectable at all. Once more, to quantitate any changes in specific RNA levels during

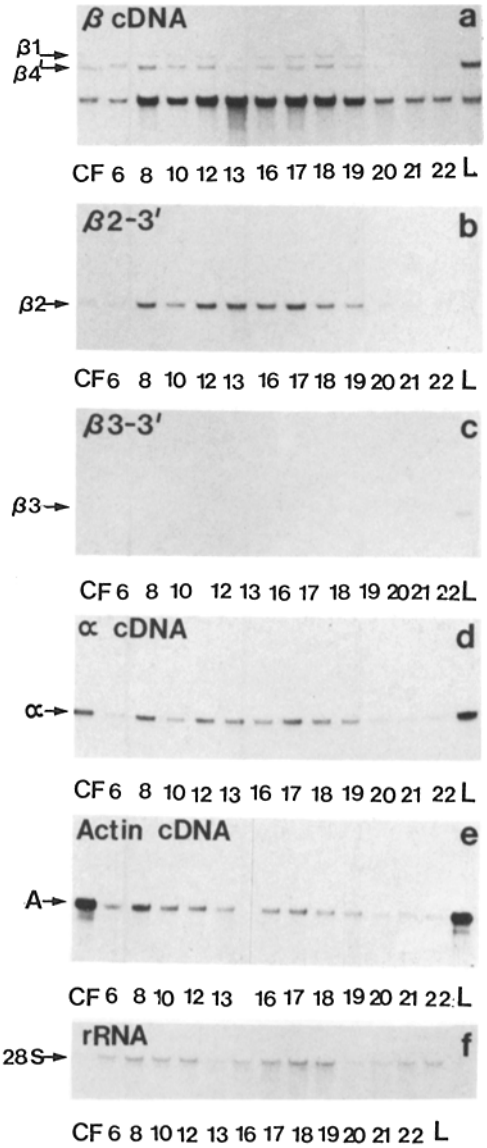

FIGURE 5 Differential expression of tubulin and actin mRNAs during chick brain development. Total RNA was isolated from embryonic chick brains from 6 d of incubation until hatching (22 d). Equal amounts of each sample (12 μg) were electrophoresed on five identical denaturing agarose gels, transferred to nitrocellulose, and hybridized to the following probes: (*a*) pT2, β-tubulin cDNA; (*b*) β2-3′ gene-specific probe; (*c*) β3-3′ gene-specific probe; (*d*) pT1, α-tubulin cDNA; (*e*) pA1, β-actin cDNA; (*f*) 28S rRNA probe. Autoradiograms of the resultant hybridizations are shown. Tracks: *CF*, secondary chick fibroblast RNA; *L*, 1104, a cloned cell line that was derived from a bursal tumor induced by avian leukosis virus infection. *6–22*, RNA from 6–22-d-old embryos.

development, all blots were rehybridized to the ribosomal probe to measure equal loading and blotting of each RNA sample. The autoradiograms from duplicate gels were then scanned densitometrically and the results plotted in Fig. 4*b*. β3 is seen to rise slowly from 5 d until hatching, whereas both transcripts from β1 remain low throughout although there is a small peak at 13 d. β4′ is relatively high at 5 d but falls slowly in amount as hatching approaches. β2 shows the greatest fluctuations increasing 15-fold from 5 d until 12 d then falling towards hatching. α-Tubulin also peaks at 12 d but shows only a fivefold increase in level. In contrast, actin RNA levels are seen to decrease sharply from 5 to 10 d and then remain at this relatively reduced level.

Tissue-specific Expression of Chicken β-Tubulins

To examine differential β-tubulin gene expression in greater detail, we isolated poly A-containing RNA from a number of adult chicken tissues. Those examined (shown in Fig. 6) included lung (lane *1*), esophagus (lane *2*), oviduct (lane *3*), liver (lane *4*), brain (lane *5*), intestine (lane *6*), spleen (lane *7*), bursa (lane *8*), thymus (lane *9*), and testis (lane *10*). Fig. 6 shows the autoradiograms of β-tubulin RNAs detected. Blots were hybridized to the β-tubulin cDNA (Fig. 6*a*), the β2-3′ (Fig. 6*b*), or β3-3′ (Fig. 6*c*) gene-specific probes. All tissues in tracks 1–7 were from the same 10-mo-old chicken. Differential β-tubulin gene expression is readily apparent and also complex (compare *a*, *b*, and *c* of Fig. 6). The most striking example of such differential expression is in testis (track *10*), where β3 is expressed overwhelmingly compared with the other three genes. β3 is also strongly expressed in oviduct (track *3*), liver (track *4*), and both bursa and thymus (tracks *8* and *9*). β2, however, is strongly expressed only in brain (track *5*) and lung (track *1*). β4′ is expressed in all tissues and is a significant transcript in liver (track *4*), spleen (track *7*), bursa (track *8*), and thymus (track *9*). Both species transcribed from β1 are minor or undetectable in all tissues examined.

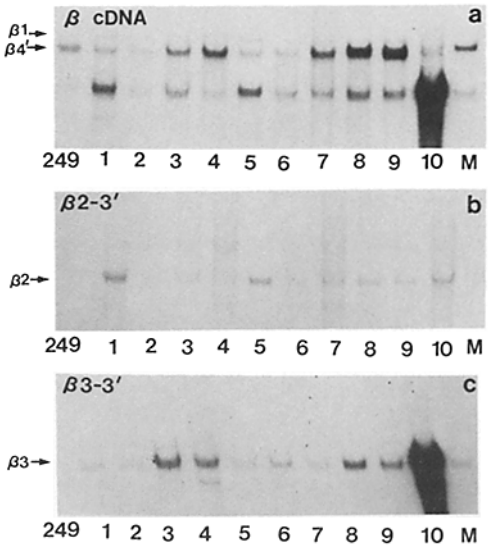

FIGURE 6 Expression of β-tubulin mRNAs in isolated chicken tissues. Poly(A)-containing RNA was isolated from a number of adult chicken tissues. Samples were electrophoresed on denaturing agarose gels, then transferred to nitrocellulose, and hybridized to the following probes: (a) pT2, β-tubulin cDNA; (*b*) β2-3′ gene-specific probe; (c) β3-3′ gene-specific probe. *249*, a clonal isolate from an MC29 virus-induced hepatoma; *1*, lung; *2*, esophagus; *3*, oviduct; *4*, liver; *5*, brain; *6*, intestine; *7*, spleen; *8*, bursa; *9*, thymus; *10*, testis; *M*, MSB, a cloned chick T lymphocyte cell line.

Cell Type-specific Expression of Chicken β-Tubulins

Inherent to the examination of RNA from whole embryos and tissues is the problem that they are composed of many cell types and the results obtained reflect an averaging of the levels of gene expression in these heterogeneous populations. Therefore, it is difficult to relate the preferential expression of any one β-tubulin gene with the specialized microtubule functions intrinsic to a particular cell type. For example, in RNA from whole brain (Figs. 5 and 6) it is unclear whether the high level of the β2 transcript is contributed by the neuronal or glial cell populations.

To investigate this kind of ambiguity, we examined the β-tubulin mRNA populations of a number of isolated cell types. Poly A-containing RNA was again electrophoresed on denaturing agarose gels, transferred to nitrocellulose, and probed either with the β-tubulin cDNA (Fig. 7*a*), the β2-3′ (Fig. 7*b*), β3-3′ (Fig. 7*c*), or β1-3′ (Fig. 7*d*) gene-specific probes. All cells were isolated from embryos except the hepatocytes, which were derived from 16-d-old chickens. The data demonstrate that, even with pure cell populations, differential β-

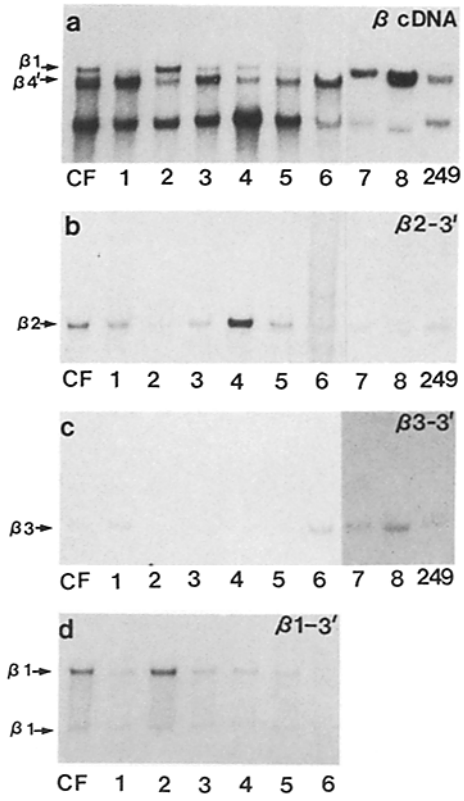

FIGURE 7 Expression of β tubulin mRNAs in isolated chicken cells. Poly(A)-containing RNA was prepared from a number of isolated cell types. Samples were electrophoresed on denaturing agarose gels, transferred to nitrocellulose, and hybridized to the following probes: (a) pT2, β-tubulin cDNA probe; (*b*) β2-3′ gene-specific probe; (c) β3-3′ gene-specific probe; (*d*) β1-3′ gene-specific probe. *CF*, secondary chick fibroblasts; *1*, chondroblasts; *2*, skeletal muscle myotubes; *3*, smooth muscle cells; *4*, spinal cord neurons; *5*, brain glial cells; *6*, hepatocytes; *7*, MSB, a cloned chick T lymphocyte cell line; *8*, primitive red blood cells; *249*, a clonal isolate from an MC29 virus-induced hepatoma.

tubulin gene expression is evident. The two transcripts from β1 are present in very low but approximately equal amounts in most cell types. The exceptions are hepatocytes (track *6*) where neither is detectable or in chick fibroblasts (*CF*) and skeletal muscle myotubes (track *2*) where there is significantly more of the 4,000-base transcript than the 1,800-base species. Overall, β1 is a minor transcript except in skeletal muscle myotubes. β2 is more prominent than β3 in all cells except primitive red blood cells (track *8*) and hepatocytes (track *6*). Moreover, it is notable that β2 is expressed more strongly in isolated spinal cord neurons (track *4*) than in the brain glial cell population (track *5*). β4′ represents a significant transcript in all cells examined and is the major species in chondroblasts (track *1*), hepatocytes (track *6*), and primitive red blood cells (track *8*).

These findings along with the corresponding data for β-tubulin gene expression in each tissue type are summarized in Table I.

Are the Polypeptides That Correspond to Each β-Tubulin Gene Biochemically Distinguishable?

To begin to test whether each β-tubulin in mRNA encodes a biochemically distinguishable polypeptide, we have translated in vitro the mRNA from β1, β2, β3, and β4′ and examined the mobility of each translation product on two-dimensional polyacrylamide gels (Fig. 8). For β1 and β4′ the source mRNAs were the gel-fractionated chicken fibroblast RNAs whose isolation and purity we have previously described (13). For β2, brain RNA, which is overwhelmingly abundant in the β2 transcript (see Fig. 5, *a* and *b*), was chosen; for β3, poly A⁺ testis RNA was utilized. Each of these four RNA species was translated separately and the translation products analyzed by two-dimensional PAGE according to the method of O'Farrell (16). All of these samples contain mRNAs coding for other proteins besides the specific β-tubulin species and these, particularly the actins, are useful in helping to align parallel gel patterns. The tubulin regions of the resultant two-dimensional gel patterns are shown in Fig. 8, *a–d* for β1–β4′, respectively. No apparent differences in isoelectric point or apparent molecular weight are observable among the four polypeptides. Moreover, co-electrophoresis of mixtures of all possible pairs of translation products also fails to reveal any detectable differences (data not shown). We conclude that the primary translation products of β1–β4′ are not distinguishable with these electrophoretic assays.

Recent work from Murphy and Wallis (21, 22) has demonstrated the presence of a β-tubulin polypeptide in chicken erythrocytes, which is markedly divergent from chick brain β-tubulin in a number of biochemical properties. These include a more basic isoelectric point, an increased electrophoretic mobility on some SDS polyacrylamide gel systems, and a surprisingly divergent primary sequence as indicated by the two-dimensional tryptic peptide maps that show ~50% of the peptides at positions unique to the erythrocyte β-tubulin. However, none of the in vitro translation products of β1, β2, β3, or β4′ display the more basic isoelectric point (Fig. 8) characteristic of this erythrocyte polypeptide. In addition, altered one-dimensional mobilities characteristic of this polypeptide are not seen (data not shown). With the caveat that we cannot be certain that the mature polypeptide isolated by Murphy and Wallis has not been posttranslationally modified

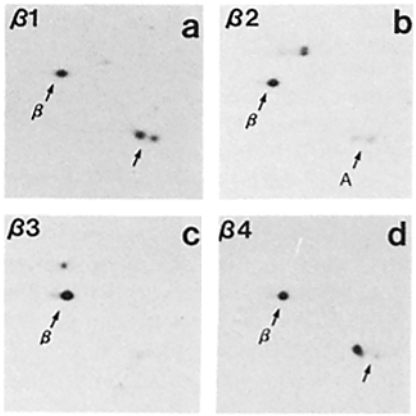

FIGURE 8 In vitro translation of mRNA from each of the β-tubulin genes. mRNA derived from each of the four β-tubulin genes was translated in vitro in the reticulocyte lysate cell-free translation system and the products were separated by two-dimensional PAGE. For β1 and β4′ the gel-fractionated chicken fibroblast mRNA previously described (13) was used as the source RNA. For β2 or β3, brain or testis RNA was used as these tissues contain overwhelmingly the β2 or β3 transcripts, respectively. The samples used contain RNAs coding for other proteins besides the specific β-tubulin species. As judged by hybridization of these mRNA fractions to the β-actin cDNA clone, pA1, the prominent actinlike proteins marked with unlabeled arrows in *a* and *d* cannot be actin. True actin spots are arrowed with an *A* in *b*, whose RNA fraction contains substantial actin sequences (data not shown). The reticulocyte translation mixture without added RNA yields no detectable proteins in the tubulin or actin regions (data not shown). (*a*) β1-tubulin; (*b*) β2-tubulin; (*c*) β3-tubulin; (*d*) β4′-tubulin.

TABLE I

Differential Expression of β-Tubulin Genes in Chicken Cells and Tissues

	β1	β4′	β2	β3	Developmental stage*
Cells					
Fibroblasts	+	+	++	+	E
Chondroblasts	+	++	+	+	E
Skeletal muscle	++	+	++	+	E
Smooth muscle	+	+	++	+	E
Neurons	+	+	+++	+	E
Glia	+	+	++	+	E
Hepatocytes		+++		+	A
Primitive red blood cells		+++		+	E
Tissues					
Lung	+	+	++	+	A
Esophagus	+	++	+	++	A
Oviduct	+	++		++	A
Liver		+++		+	A
Brain (adult)	+	+	++	+	A
Brain (embryo)	+	+	++++		E
Intestine		+++		++	A
Spleen		+++	+	+	A
Bursa		+++		++	A
Thymus		+++		++	A
Testis		+		++++++	A

The data is taken from the autoradiograms displayed in Figs. 5 and 6.
* E indicates that the cell or tissue was isolated from an embryo. A indicates that the cell or tissue was isolated from an adult.

in some important manner, these data indicate that none of our isolated genes encodes the prominent erythrocyte β-tubulin.

DISCUSSION

In this work we have used probes specific to individual chicken β-tubulin genes to monitor the levels of different β-tubulin mRNAs during the development of whole chick embryo and embryonic brain and in a number of chicken cell and tissue types. The results of our survey of β-tubulin expression are summarized in Table I. What can be clearly be seen from these data is that the pattern of β-tubulin gene expression is complex, although certain significant correlations can be made. In particular:

Development—General

At all embryonic stages examined all four β-tubulin genes are active although they show dramatic differences in their relative levels of expression and some changes in level as a function of development.

Development—Neural

(*a*) In developing brain there are major differences in the levels of expression among the four β-tubulin genes although these are distinct from those occurring in the whole embryo.

(*b*) We do not detect the appearance of a new size class of β-tubulin mRNA (even on very long exposures) as has been documented for the β-tubulins in developing rat cerebellum (24).

Tissue and Cell Type Differences

(*a*) At least two, and often three or four β-tubulin genes are expressed in each cell and tissue type examined.

(*b*) The level of expression of individual genes in each cell type does not appear to correlate with the embryonic layer of origin of that cell type.

(*c*) β2 is more prominent than β3 in all embryonic cells or tissues examined except primitive red blood cells whereas β3 is more strongly expressed than β2 in all adult-derived cells and tissues except brain and lung.

(*d*) β4′ is expressed in all cells and tissues examined and therefore may be constitutive, serving a ubiquitously required tubulin function.

(*e*) β1 is a minor or undetectable transcript in all but isolated skeletal muscle myotubes. Possibly this reflects a specialized function for this β-tubulin gene. In addition, the relatively high level of expression of β1 in muscle is accompanied by a shift in the site of polyadenylation of the β1 transcript. The significance of this shift is unknown.

(*f*) β2 is strongly expressed in brain and in isolated neurons but not in glial cells. This implies that β2 encodes the dominant neuronal polypeptide, but this cannot be its exclusive function as it is also highly expressed in most embryonic cells and tissues and also in the adult lung.

(*g*) β3 is overwhelmingly the dominant β-tubulin expressed in testis, which implies a function in the formation of the sperm tail flagellum and/or in the meiotic spindle. In *Drosophila*, a β-tubulin polypeptide expressed only in testis has been identified genetically to be multifunctional, participating in assembly of both spindle and sperm tail flagellar microtubules (8). Although the chicken β3 gene is similar to this *Drosophila* β-tubulin polypeptide in that it is the dominant β found in testis, the chicken β3 gene cannot be simply equated with this *Drosophila* β because it is not expressed exclusively in testes. Rather, it is found in many other tissues, particularly in the adult.

It appears that none of our four isolated genes encodes the divergent erythrocyte β-tubulin reported by Murphy and Wallis (21, 22). Although we have recently cloned an additional segment of chicken DNA that hybridizes only weakly to the other four cloned genes, this segment (named β5 [25]) also appears not to be the progenitor of the erythrocyte β-tubulin polypeptide. This raises the intriguing question of which β-tubulin gene does encode this polypeptide. We would suggest that it is likely that the substantial sequence divergence indicated for this β-tubulin by tryptic peptide mapping could reduce the level of cross-hybridization of the corresponding gene with our cloned probes to an undetectable level. This surprising lack of cross-hybridization within the chicken β-tubulin gene family emphasizes that the analysis of gene families by hybridization techniques relies on the implicit assumption that all family members retain significant nucleic acid homology. This, of course, need not (and may not in the present example) be the case.

We have not been able to determine whether the 3,500-base β4′ RNA derives from the cloned β4 gene or from a putative, closely related sister gene, although the bulk of our present evidence favors the latter. If this scenario is correct, what then is the status of the cloned β4 gene itself? The combination of DNA sequence data and S1 nuclease protection experiments has shown that the cloned β4 gene is transcribed into a low-abundance 1,800-base mRNA that encodes a strikingly divergent β-tubulin polypeptide (26). Because we have been unable to detect this β4 mRNA by the less sensitive Northern blot technique utilized here, we have not been able to determine the program of expression of this divergent β-tubulin polypeptide.

In any case, it is clear from the data presented here that differential expression of β-tubulin genes does occur. But how does this relate to the important underlying question of the physiological significance of multiple β-tubulin genes/polypeptides in a single organism? Two possibilities must be considered. First, multiple β-tubulin genes may have evolved to allow different gene control mechanisms to operate during the course of differentiation to regulate the proper level of β tubulin polypeptides. In this model the β-tubulin polypeptides encoded by the four genes would be functionally indistinguishable or differ only in minor aspects. Expression of different β-tubulin genes could then be linked with other coordinately regulated genes that are co-activated during a specific developmental program. In particular, one can imagine that an individual β-tubulin gene might be linked in this fashion with a given α-tubulin gene, with the genes that encode microtubule-associated proteins or with genes for other proteins which interact with β-tubulin.

The alternative model is that multiple β-tubulin genes have evolved to provide functionally distinct β-tubulin polypeptides. In its ultimate form, this possibility would predict that each β-tubulin polypeptide would polymerize into microtubules containing only a single β-tubulin species, thereby conferring to the resultant microtubule a specific cellular function. A less extreme possibility would be the co-polymerization of multiple β-tubulin polypeptides into 'hybrid' microtubules in which the differential functional properties of each subunit would be manifested through preferential binding of

subunit-specific microtubule-associated proteins, etc. This latter alternative embodies the multi-tubulin hypothesis proposed initially by Fulton and Simpson (4). At present, two lines of evidence in higher eukaryotes support this hypothesis. First, our current data indicate that at least two and often three or four β-tubulin genes are simultaneously expressed within individual cell types. (We presume that these cell types actually contain the corresponding number of polypeptides although this has not been shown directly.) Nucleotide sequence analysis from this laboratory of the β2, β3, and β4 tubulin genes (20, 25, Sullivan, K. F., and D. W. Cleveland, manuscript submitted for publication) indicates that these genes do in fact encode surprisingly different β tubulin polypeptides. A second line of evidence is that Thompson et al. (26), using a monoclonal antibody raised against sea urchin axonemes that recognizes an as yet unknown determinant present on one or more α-tubulin polypeptides, have demonstrated that this antigenic determinant is available for antibody binding only in a distinct subset of the microtubules in PtK2 cells. Hence, the possible presence of different classes of microtubules constructed from different subunits within a single cell is a very real one. Moreover, the potential remains that posttranslational modification of the different gene products may also play an important role in tailoring subunits for specialized microtubule functions as has been demonstrated for flagellar α-tubulin in *Chlamydomonas* (27–29) or in α-tubulin in the rat optic nerve (30). If this is in fact the case, the possible number of different β-tubulin polypeptides and of their corresponding microtubules in the chicken could rise from the number of known functional genes to a substantially larger number.

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