Sequence of a Highly Divergent β Tubulin Gene Reveals Regional Heterogeneity in the β Tubulin Polypeptide

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ABSTRACT The nucleotide sequence of a chicken genomic DNA segment containing the chicken β4 tubulin gene has been determined. The predicted amino acid sequence of β4 is surprisingly divergent from that of the chicken β2 gene that encodes the dominant neural β tubulin. β4 differs from β2 at 36 residue positions and encodes a polypeptide that is four amino acids longer, yielding a divergence of 8.9% between the two β tubulin isotypes. While many of the amino acid substitutions are conservative, several involve significant alteration in the physiochemical properties of the residue. Furthermore, the amino acid substitution positions are not randomly located within the primary sequence but are distinctly clustered: major divergence occurs in the carboxy-terminal region beyond residue 430 and within the second protein coding exon segments of the genes. In addition, large regions of absolute sequence conservation are also present. Certain sequences within the heterogeneous regions are conserved in other species, indicating that these regions are under positive evolutionary selection pressure and are therefore probably essential for some aspect of β-tubulin function. These findings strongly suggest that regional amino acid sequence heterogeneity may play an important role in the establishment of functionally differentiated β tubulin polypeptides.

Microtubules, composed principally of dimeric subunits of 1 α and 1 β tubulin polypeptide, are filamentous polymers that are employed in a number of diverse functions in eucaryotic cells. For example, microtubules are the major components of mitotic and meiotic spindles and of cilia and flagella. Furthermore, they participate in various types of intracellular transport and, in conjunction with intermediate filaments and actin filaments, establish the structure and functional properties of the eucaryotic cytoskeleton. Yet in spite of this functional diversity, the ultrastructure of microtubules is, with a few notable exceptions (6, 10), identical in all cells. Moreover, the tubulin polypeptides themselves have been conserved throughout evolution to such an extent that tubulins from all sources investigated have been shown to co-polymerize efficiently in vitro (see reference 18 for review).

Despite these apparently conserved features, several lines of evidence suggest that the microtubule systems of different cells or within single cells are distinguishable on the basis of physical, biochemical, and pharmacological properties. For example, Benke and Forer (2) originally reported evidence for four classes of microtubules in individual cells which differed in their drug sensitivities or thermal stability. Similarly, Brinkley and Cartwright (5) have demonstrated that the interpolar and kinetichore microtubules of mammalian spindles differ in their response to drug- and cold-induced depolymerization. Additional compelling evidence has come from the work of Thompson et al. (31) and Cumming et al. (9) who have independently demonstrated, using monoclonal antibodies to α tubulin determinants, that α tubulin related epitopes are apparently segregated into distinguishable subsets of microtubules within single cells or subsets of cells. Thus, it seems clear that although microtubules appear morphologically identical, they represent a heterogeneous class of structures.

One obvious potential level of control of microtubule properties is at the level of the tubulin dimers which constitute the major structural subunit. This hypothesis would require that multiple, functionally distinct tubulin subunits be present within cells. Immunological and biochemical support for this hypothesis has emerged from a number of investigators who have reported heterogeneous pools of α and β tubulins in a number of tissue and cell types (e.g., references 11, 12, 22, 24, 25, 30) and within single cells (13). More definitively, protein sequence analysis of porcine brain tubulins has demonstrated the presence of two β tubulins (19) and four α tubulins (27) that differ subtly in primary sequence. More recently, sequence analysis of two different human β tubulin genes has demonstrated the presence of at least two β tubulin isotypes that differ significantly in sequence at the carboxy terminus of the polypeptide (15).

We previously demonstrated that the chicken contains four

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β tubulin genes that possess strong homology to a cloned β tubulin cDNA, each of which has been isolated by molecular cloning and designated β1–β4 (20). Recent experiments (Havercroft, J. C., D. B. Murphy, and D. W. Cleveland, unpublished results) indicate the presence in the chicken genome of 3–5 additional divergent β tubulin sequences with only weak nucleic acid homology to other cloned β tubulin sequences. Overall, this suggests that the chicken genome possesses not more than eight or nine functional β tubulin genes. With the goal of determining the number and identity of tubulin polypeptides that constitute a complete repertoire of tubulins necessary for the development and differentiation of a vertebrate organism, we have begun nucleotide sequence analysis of the β tubulin gene family in the chicken. We report here the sequence of the chicken β4 gene. We find that this gene encodes a polypeptide that is strikingly divergent from any previously reported vertebrate β tubulin. Comparison of these data with other currently available β tubulin sequences demonstrates the presence of regions of heterogeneity embedded within an otherwise highly conserved β tubulin polypeptide.

MATERIALS AND METHODS

DNA Sequence Analysis: DNA sequence analysis was carried out by the chain terminator method of Sanger et al. (28) using fragments of the cloned pβG4 gene which had been subcloned into the Messing M13 vectors (23), mp8 or mp9. Random fragments of the plasmid pβG4 were generated by digestion of the plasmid DNA with DNAse I (Worthington Biochemical Corp, Freehold, NJ) in the presence of Mn^{+2}, as described by Anderson (1). Briefly, 100 μg of supercoiled plasmid pβG4 DNA (19) was digested with 5 μg DNAase I in the presence of 1 mM $MnCl_2$ at room temperature and 25 μg samples were removed after 12, 15, 18, and 21 min of reaction and stopped by addition of 10 mM EDTA. Samples were pooled and, after deproteinization, ends were repaired by fill in with DNA polymerase I, Klenow fragment (Bethesda Research Laboratories, Gaithersburg, MD). The DNA was resolved on a polyacrylamide gel and fragments in the range from 600–2,000 bp were collected by electroelution. Recovered fragments were then ligated with M13 mp8, which had been cut with *HincII* and treated with calf intestinal phosphatase, and transformed into JM101. Phage plaques were then screened by the method of Benton and Davis (3) using the 5.3 kb *XhoI-KpnI* fragment of pβG4 (see Fig. 1*A*) which had been labeled with ^{32}P using the method of Shank et al. (29). DNA was then prepared from positive plaques for sequencing. Some fragments were also obtained by directed cloning of restriction fragments into the appropriate double cut M13 vectors.

S1 analysis: S1 nuclease protection experiments were performed by a modification of the method of Berk and Sharp (4). To probe for the 3′ end of β4 transcripts, the 2.5 kb Bam H1 fragment of pβG4 was isolated by agarose gel electrophoresis and labeled at 3′ ends by incubation with DNA polymerase I Klenow fragment in the presence of α-^{32}P-dATP (400 Ci/mmol, Amersham Corp., Arlington Heights, IL) and 1 mM each dGTP, dCTP, and dTTP for 15 min at 30°C. Messenger RNA (mRNA) was prepared from whole 7-d chicken embryos using the guanidinium thiocyanate procedure of Chirgwin et al. (7). Probe (0.01–0.4 μg) was co-precipitated with 0.25 to 1.0 μg of mRNA plus 400 μg of carrier yeast tRNA and then redissolved in 20 μL of a solution containing 80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, and 1 mM EDTA. Samples were incubated at 85°C for 10 min to denature the probe and then at 60°C for 3 h to allow hybridization to occur. Hybridization mixtures were then diluted with 200 μL of S1 reaction buffer containing 500 U of S1 nuclease (Bethesda Research Laboratories) and reactions were incubated at 22°C for 60 min. Samples were then prepared for electrophoresis and resolved on 6% polyacrylamide sequencing type gels and results were visualized by autoradiography.

RESULTS

Of the four chicken β tubulin genes that we have cloned, we have previously determined the coding region sequence for the chicken β2 gene, the genomic progenitor of the full length cDNA clone pT2 (8, 32). This gene encodes the dominant neuronal β tubulin of the chicken (16, 20). To begin to analyze the diversity of β tubulin polypeptide sequences encoded within this organism we have now determined the sequence of the β4 gene. This gene was elected for analysis because RNA and DNA blot hybridization experiments had shown that it was divergent from the β2 sequence (20). Plasmid pβG4 containing the β4 gene subcloned into the *SphI* site of pBR322 (as described previously [20]) is illustrated in Fig. 1 *a*. The 5.3 kb *XhoI-KpnI* fragment of pβG4 contains the entirety of the sequences homologous to gene β2. This fragment was subjected to shotgun DNA sequence analysis using M13/dideoxy sequencing techniques as described in Materials and Methods. The sequences of 71 subcloned fragments, representing a total of 12584 bases, were used to assemble the final sequence. Overall, we have determined the sequence of 5358 contiguous bases spanning the entire *XhoI-KpnI* fragment. Greater than 90% of the sequence was read from both strands or from multiple overlapping clones; the actual sequencing strategy is shown in Fig. 1 *b*.

The structure of the β4 gene as determined by comparison with the known coding region sequence from β2 is diagrammed in Fig. 1 *c*. The organization is identical to that of the human β tubulin gene M40 (14) and consists of four blocks of protein coding exon sequences interrupted by three intervening sequences placed between codons 19 and 20, and within codons 56 and 93, respectively. Thus, it appears that the coding structure of β tubulin genes has been conserved throughout vertebrate evolution. A putative RNA polymerase II promoter element, TATAAA, is located 100 bases 5′ to the initiator ATG codon and a consensus polyadenylation signal, AATAAA, is located 165 nucleotides 3′ of the TGA translation termination codon. No additional polyadenylation signals were found in the 2.3 kb of 3′ flanking DNA whose sequence was determined.

Amino Acid Sequence of the β4 Polypeptide

From the nucleotide sequence we have deduced the predicted protein sequence of the polypeptide encoded by the β4

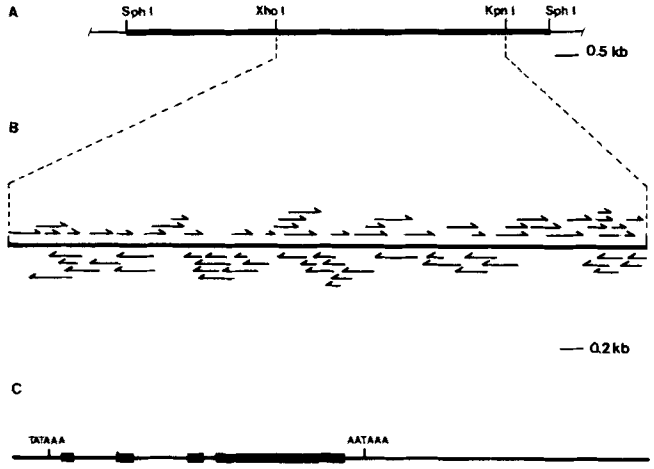

FIGURE 1 Organization, sequencing strategy, and structure of the chicken β4 tubulin gene. (*A*) The organization of the recombinant plasmid pβG4 (20) which contains a 10-kb fragment of chicken genomic DNA cloned into the *Sph 1* site of pBR322. The 5.3 kb Xho I-Kpn I fragment, which contains the entire β4 coding region and 2.5 kb of 3′ flanking sequences was chosen for DNA sequence analysis. (*B*) Schematic drawing of the fragments used to assemble the β4 sequence. (C) The structure of the β4 gene. Filled boxes denote protein coding exon sequences while noncoding sequences are shown by the thin line. The positions of putative transcription control sequences, the *TATAAA* promoter, and *AATAAA* polyadenylation site are noted. The direction of transcription is from left to right.

gene. The relevant DNA sequence and corresponding translation are shown in Fig. 2, along with the previously determined coding sequence of the dominant chicken neural β tubulin gene, $\beta 2$. It is readily apparent that the $\beta 4$ gene encodes a polypeptide that is surprisingly divergent compared with the dominant neural β tubulin. There are a total of 36 amino acid substitutions between these two proteins. In addition, the predicted $\beta 4$ polypeptide is four amino acids longer than the prototypic $\beta 2$ polypeptide. Most striking is the presence of a basic lysine residue that terminates the highly acidic carboxy-terminus of the protein. Thus, the overall divergence between these two proteins is 8.9%: this is to be compared with 1 and 5% divergence noted between the $\beta 2$ sequence and the sequence of a porcine brain (19) or human (15) β tubulin, respectively.

Comparison of the $\beta 4$ and $\beta 2$ Polypeptide Sequences

Detailed comparison of the amino acid differences between the $\beta 2$ and $\beta 4$ polypeptides shows generally conservative amino acid substitutions. Several substitutions, however, involve significant changes in the chemical properties or size of the side chain. Three substitutions, at residues 37, 53, and 83, involve exchange of aliphatic or amide-containing residues for histidine, resulting in a net gain of two histidine residues in $\beta 4$. One marked change is $\beta 2$ Ser 275 to $\beta 4$ Arg 275, a change that involves not only the acquisition of an additional fixed positive charge in $\beta 4$, but also a significant alteration in the size of the side chain. Curiously, there is a complementary exchange of serine and cysteine residues in positions 124 and 239, substitutions likely to significantly alter the chemical properties of these residues. However, since these changes involve an exchange of serine and cysteine residues, it is tempting to speculate that these residues may be close to each other in the folded polypeptide and that the exchange preserves a functional requirement for the cysteine residue.

The major region of heterogeneity between $\beta 2$ and $\beta 4$ is localized to the extreme carboxy terminus of the proteins, including an additional four-amino acid extension for the $\beta 4$ polypeptide. Most of the substitutions in this region involve exchange or acquisition of acidic residues in $\beta 4$, preserving the overall net charge of this region. Two striking substitutions are found, however, replacing the peptide pair Phe436-Glu437 in $\beta 2$ with Met-Tyr in $\beta 4$. In addition, the four residue extension sequence, Gln-Gly-Ala-Lys, which in addition to

FIGURE 2 Comparison of the coding region of $\beta 4$ with the coding region of $\beta 2$ (pT2), the dominant chicken neural β tubulin. The coding sequence of $\beta 4$ is shown with the corresponding predicted translation to protein sequence above it. The three intron sequences, inserted between codons 19 and 20 and between bases 1 and 2 of codons 56 and 93, respectively, are not shown. The sequence is aligned with the nucleotide sequence of the full length β tubulin cDNA clone pT2 (32) which is derived from the chicken $\beta 2$ gene; identical nucleotides are denoted by dashes while nucleotide differences are explicitly shown. Amino acid residues of $\beta 2$ which differ from $\beta 4$ are noted beneath the $\beta 2$ nucleotide sequence.

the terminal lysine is comprised of small neutral amino acids, represents a significant departure from the highly acidic carboxy-terminal sequence that preceeds it.

Regional Heterogeneity within β Tubulin Polypeptides

The amino acid substitutions between the β2 and β4 polypeptides do not appear to be homogeneously distributed, but rather are clustered within the primary sequence, giving rise to regions of high sequence heterogeneity as well as regions of high conservation. This perhaps best illustrated in Fig. 3 in which we have presented the parallel comparison of all currently available vertebrate β tubulin sequences. Immediately apparent in this comparison is the prominent sequence divergence among these various β tubulins in the carboxy terminal region beyond amino acid 431 (see also discussion above). In particular, this region accounts for 33% of the divergence between β4 and β2 and also represents a major variable region between two human β tubulin genes (15). Moreover, a second variable region in which substantial divergence is also obvious is found between residues 33 and 57. This 24-amino acid portion of the polypeptide possesses seven substitution positions between β4 and β2. Inspection of Fig. 3 shows that this region is also a site of significant microheterogeneity between porcine brain β tubulins (19). Although most of the substitutions in this region are conservative, two substitutions involve exchange of neutral residues for histidine (as detailed above).

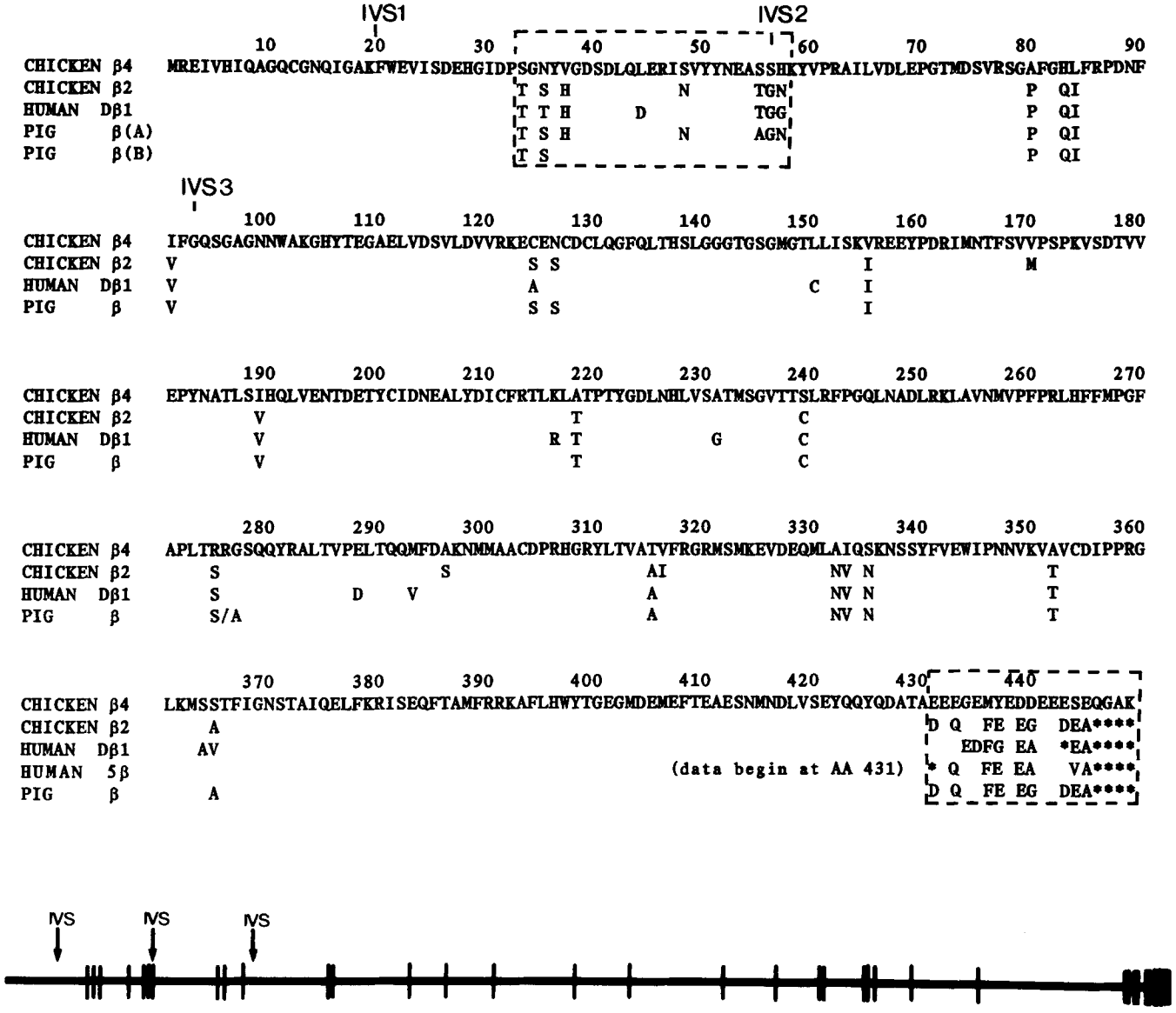

FIGURE 3 Comparison of the chicken β4 tubulin sequence with the sequences of other vertebrate β tubulins. The sequence of the predicted chicken β4 tubulin polypeptides is shown (using the one letter amino acid code) and has been aligned with the predicted protein sequences of the chicken β2 tubulin gene (32), two human β tubulin sequences, Dβ1 and 5β (obtained by DNA sequencing of a fetal human brain cDNA clone and a partial sequence of a functional human β tubulin gene, respectively [reference 15]), and two variants, *A* and *B*, of porcine brain β tubulin (19). With the exception of residue position 275, porcine variants *A* and *B* differ only within the first 83 residues (the first line of the figure) and are therefore shown as a single sequence on subsequent lines. In each of the other sequences, only positions that differ from the chicken β4 sequences are noted. The known sequence for human 5β begins at residue 431. Dashed boxes enclose the regions of highest sequence divergence as discussed in the text. The diagram below shows the linear distribution of amino acid substitution positions between chicken β2 and β4 tubulins (vertical dashes) shown within the primary sequence of β tubulin (horizontal line).

In addition to regions of heterogeneity, regions of extreme sequence conservation are also strikingly evident in the comparison presented in Fig. 3. For example, the amino terminal portion up to residue 32 is invariant among the known vertebrate sequences, as is the carboxy terminal region between residues 366–431.

A summary of these observations is presented in the line drawing at the bottom of Fig. 3 which shows the linear distribution of amino acid substitutions between the chicken β4 and β2 tubulin isotypes. Overall, the data suggest that the β tubulin polypeptide is organized so as to incorporate regional heterogeneity within a highly conserved framework.

β4 Gene Is Expressed during Chicken Development

The β4 gene thus encodes a polypeptide with surprisingly divergent primary sequence. But is this predicted β tubulin subunit actually expressed and used in cellular microtubule systems? Blot analysis of mRNA transcripts of the chicken β tubulin genes has previously demonstrated that the β4 genomic sequence hybridizes strongly and preferentially to a 3,500-3,700 base mRNA present at high abundance in most tissues and cell types tested (16, 20), thus suggesting that the β4 gene product is a ubiquitous and perhaps constitutive β tubulin subunit. However, this initial blot data has proven to be misleading. Contrary to our initial expectations, more recent work has shown that neither the immediate 5′ or 3′ flanking regions of the β4 gene are present on the 3,500–3,700 base β tubulin mRNA (16). In addition, the present sequence data demonstrate the presence of a consensus polyadenylation signal, AATAAA, 165 bp 3′ to the termination codon, and a putative promoter element, TATAAA, located 100 bases 5′ to the ATG translation initiation codon. Assuming both that transcription begins 30 bases downstream of the TATAAA sequence (reviewed, for example, in reference 26) and the presence of a 200 nucleotide 3′ poly-A tract beginning 20 bases downstream of the polyadenylation signal, these data predict an mRNA species of ~1,800 bases, markedly different from the previously detected 3,500–3,700 base mRNAs. No additional polyadenylation sites are present in the 2.3 kb of downstream flanking sequence.

To determine whether the β4 gene is a functional and expressed gene, we performed the following S1 nuclease protection experiment. A 2.3-kb fragment that begins in the coding region within codon 344 and extends through 317 bp of the *C*-terminal coding region and terminates in the flanking DNA ~2 kb 3′ to the translation termination codon, was prepared from pβG4 by Bam H1 digestion. This fragment was labeled with ^{32}P at 3′ ends as described in Materials and Methods and hybridized to poly-A+ mRNA from 7-d chick embryos. After hybridization, the mixture was digested with S1 nuclease to remove unhybridized single stranded regions of DNA, and the labeled fragments protected from digestion by hybridization to RNA were analyzed by electrophoresis and autoradiography as shown in Fig. 4. Clearly, the predominant mRNA species, which is homologous to the β4 gene probe, protects a fragment ~510 bases in length. This is precisely the length of protected DNA expected if the polyadenylation signal sequence 165 bases downstream of the termination codon is used. Furthermore, there is no evidence for the presence of a larger RNA transcript that derives from this β4 gene.

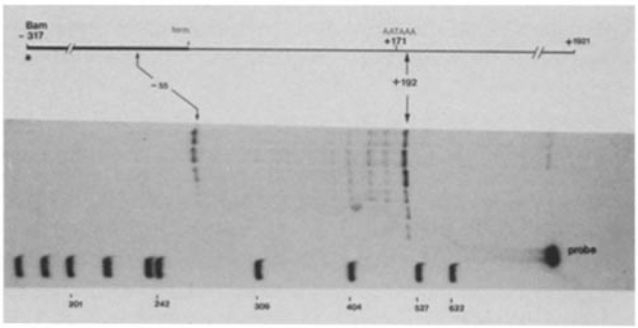

FIGURE 4 S1 nuclease protection analysis of β4 gene transcripts. An S1 nuclease protection experiment was performed as described in Materials and Methods using the 2.5 kb Bam H1 fragment of pβG4 which spans the 3′ end of the gene. This fragment is diagrammed at the top of the figure and extends 317 bp 5′ of the TGA translation termination codon to 1921 bp 3′ of the termination codon. The position of the final A of the polyadenylation signal, 171 bp 3′ of the termination codon, is also noted. The autoradiogram is oriented with the bottom of the gel at left; the experimental lanes shown represent a titration of probe DNA from 12.5 to 400 ng in a series of twofold increments. Probe alone is noted in the next to the last lane and pBR322/Hpa II size markers are shown in the bottom lane. The positions of the two major bands are noted with respect to the sequence; the numbering refers to the size of the fragment with respect to the translation termination codon.

This experiment unambiguously demonstrates that the β4 gene is transcribed into a polyadenylated mRNA. However, this mRNA represents a minor component of the β tubulin mRNA transcripts in most cells and tissues as we have not to date detected significant quantities of the β4 transcript by RNA blot analysis using β4 specific 5′ or 3′ untranslated region probes (16). Curiously, however, the experiment shown in Fig. 4 demonstrates the presence of a second, shorter protected fragment, corresponding to extensive nucleic acid homology with an mRNA that extends only up to amino acid residue 430 (~55 bases 5′ to the stop codon). This protected fragment must arise from hybridization to an mRNA that is transcribed from a gene extremely homologous to, but not identical to, the cloned β4 gene. From the known sequences (32; Sullivan and Cleveland, unpublished results), RNAs transcribed from the chicken β1, β2, and β3 genes are unlikely to generate this protected fragment since for each of these the major region of sequence divergence from β4 lies near residue 351 rather than near position 430. Therefore, we suggest that the mRNA which gives rise to the shorter protected probe is probably the 3,500–3,700 base transcript detected with overwhelming preference by the β4 gene in blotting experiments and that this mRNA derives from a β4-like gene that we have not yet cloned.

In any case, irrespective of the surprisingly complex relationship between the chicken β4 and β4-like tubulin gene, it is clear that the cloned β4 gene does represent an authentic, functional β tubulin gene that is transcribed into a stable mRNA encoding a highly divergent β tubulin subunit.

DISCUSSION

Previous studies of tubulin polypeptide structure have stressed the conservation of β tubulins throughout evolution. For example, the partial sequence data of Luduena and Woodward (21) initially demonstrated that the amino termini of sea urchin flagellar and chicken brain tubulins were idential. More definitively, subsequent comparison of the entire se-

quence of a chicken brain cDNA for β tubulin (32) and porcine brain β tubulin (19) showed a conservation of 99% in the primary sequences of these two polypeptides. Although Krauhs et al. (19) identified sequence microheterogeneity between two procine β tubulins, this heterogeneity was restricted to six residue positions, suggesting that the differences between different β tubulins were quite subtle. Together, these previous studies indicated that β tubulin was among the most highly conserved proteins known.

It has now become clear that this conclusion is both correct and misleading. Not only has the sequence of a human β tubulin (Dβ1) demonstrated a much lower degree of conservation (15), we have now demonstrated a level of isotypic variation of β tubulins within a single species of 40 of 449 residues (8.9%). This level of divergence is greater than that seen in any interspecies comparison of available metazoan β tubulin sequences. However, certain isotypic forms of β tubulin, for example the neuronal polypeptides represented by the β2 sequence and the porcine variant A, have remained highly conserved during evolution. Thus, while the individual isotypes that constitute the β tubulin family have diversified *within* species during evolution, individual isotypic forms of β tubulin have been highly conserved *between* species.

Collectively, it appears certain from the data currently available that the expression of different β tubulin isotypes is a general property of metazoan species. But are the isotypic differences which distinguish different tubulins within a single species functionally meaningful? Kemphues, Raff, and coworkers (17) have presented an elegant genetic and morphological analysis of a series of mutants in a testes specific β tubulin variant and have demonstrated that this unique protein is the major β tubulin subunit of postmitotic spermatids and that it apparently functions in all postmitotic microtubule systems of the spermatid. Documentation of this multifunctional behavior presents a compelling argument against a unique testes specific function for this β subunit. This has led these workers to suggest instead that multiple tubulin genes may be functionally equivalent, representing duplicated genes that have evolved into different genetic regulatory systems, thereby enabling the cell to coordinate tubulin synthesis with specialized programs of differentiation.

However, the sum of the presently available sequence data (Fig. 3) offers equally compelling molecular evidence for the alternative hypothesis of different, functionally specialized β tubulin gene products. Many of the differences that distinguish the two chicken β tubulin isotypes are distinctly clustered between regions of the polypeptide that are remarkably conserved among other known vertebrate β tubulins. Of course, it may be argued that variable regions simply represent nonessential portions of the molecule that are not subject to strong selection at the level of protein sequence. This hypothesis seems unlikely, however, since it is clear from inspection of the interspecies comparisons in Fig. 3 that certain sequences within the clusters of divergence have been highly conserved during evolution. For example, the chicken β2 sequence is virtually identical to procine brain β variant A, even in the regions of high substitution frequency. It seems likely therefore that the conserved sequence exemplified by β2 represents the predominant neural β tubulin isotype found in each of these organisms. Furthermore, several of the amino acid substitutions that characterize the amino-terminal variable cluster of β4 are also observed in the porcine brain variant β (19). Since this area is entirely encoded on exons 2 and 3 of the β tubulin genes, it is plausible that it constitutes a variable domain within the β tubulin polypeptide.

Since the specific sequence arrangements found in the heterogeneous regions cannot be essential for the highly conserved functions of β tubulin (polymerizability, etc.), the observed conservation of sequences in tubulins from different species argues strongly that the heterogeneous regions have been maintained by evolutionary selection for specific functional properties. An alternative, but perhaps less likely, explanation is that the variable sequences have not been conserved, but have undergone convergent evolution to similar sequences in response to similar structural requirements that have arisen during evolution. Both explanations, however, imply that specific sequence arrangements in the variable regions are the result of positive selective pressure. We cautiously suggest that the conservation of variable region sequences in β tubulin polypeptides forms the foundation of a convincing demonstration of a functional role for the variable domains, a suggestion that can be directly tested by further sequence analysis of β tubulins from different organisms.

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