The EMBO Journal vol.7 no.4 pp.931 - 940, 1988

A survey of the α-tubulin gene family in chicken: unexpected sequence heterogeneity in the polypeptides encoded by five expressed genes

Lisa F.Pratt[1] and Don W.Cleveland

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205, USA

[1]Present address: Division of Clinical Immunology, Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

Communicated by K.Weber

To characterize the α-tubulin gene family in chicken, we have isolated five chicken α-tubulin genes and determined the majority of the sequences of the encoded polypeptides. Three of these (cα3, cα5/6 and cα8) encode novel, expressed α-tubulins that have not previously been analyzed, whereas one gene segment is a pseudogene and another appears capable of encoding a functional subunit (although we were unable to document its expression in a survey of chicken tissues). Together with two additional expressed, functional α-tubulins reported earlier, we conclude that the chicken α-tubulin family is comprised of at least five functional genes whose polypeptide products are substantially more heterogeneous than found in preceding analyses of vertebrate α-tubulins. Comparison of the amino acid sequences reveals that the five polypeptides are between 96 and 83% identical, with the extreme carboxy-terminal residues representing a highly heterogeneous variable domain. Since some α-tubulins undergo cyclic post-translational removal and readdition of a carboxy-terminal tyrosine, the notable sequence divergence in this domain suggests that individual tubulins probably participate to different extents in this modification cycle.

Key words: α-tubulin/chicken/gene family/polypeptide sequence heterogeneity

Introduction

In the 16 years since α- and β-tubulin were first determined to be the principal subunits of eukaryotic microtubules, it has become abundantly clear that these polymers contribute at least two essential functions to all higher eukaryotes. First, microtubules are responsible for accurate chromosome segregation at cell division. Second, in concert with actin filaments and intermediate filaments, microtubules play a major role in establishing and maintaining the dynamic spatial organization of the cytoplasm (Heuser and Kirschner, 1980). In addition, microtubules also participate in a spectrum of specialized roles as major components of eukaryotic cilia and flagella (Gibbons, 1975), in establishing the highly asymmetric morphology of neurons, and in transport of vesicles and organelles within the cytoplasm (Hayden and Allen, 1984; Vale *et al.*, 1985).

Analysis of the molecular genetics of tubulin has lead to recognition that in higher eukaryotes small multigene families encoding both α- and β-tubulin lay beneath this heterogeneity of microtubule utilization (see Cleveland and Sullivan, 1985; Cleveland, 1987). For β-tubulin, extensive analyses in chicken (Sullivan and Cleveland, 1984; Sullivan *et al.*, 1985, 1986a,b; Murphy *et al.*, 1987; Montiero and Cleveland, 1988), mouse (Lewis *et al.*, 1985a; Sullivan and Cleveland, 1986; Wang *et al.*, 1986) and human (Hall *et al.*, 1983; Lee *et al.*, 1983; Lewis *et al.*, 1985b; Sullivan and Cleveland, 1986) have revealed six functional vertebrate β-tubulins. At least four [and probably five—see Lopata and Cleveland (1987)] of these represent distinct β-tubulin isotypes whose sequences (particularly within a carboxy-terminal variable domain) have been evolutionarily conserved (Sullivan and Cleveland, 1986; Wang *et al.*, 1986).

For α-tubulin, the analyses are less complete. In part this is the consequence of an abundant number of tubulin pseudogenes that are present in mammalian genomes (e.g. Lemischka and Sharp, 1982; Wilde *et al.*, 1982; Lee *et al.*, 1983). In fact, despite reports of cDNA clones for several α-tubulins (Cleveland *et al.*, 1980; Ginzburg *et al.*, 1981; Lemischka *et al.*, 1981; Cowan *et al.*, 1983; Lewis *et al.*, 1985a; Elliott *et al.*, 1986; Villasante *et al.*, 1986; Dobner *et al.*, 1987; Pratt *et al.*, 1987), analysis of only one rat α-tubulin (Lemischka and Sharp, 1982) and two human α-tubulin (Hall and Cowan, 1985; Dobner *et al.*, 1987) genes have been reported. To surmount such problems, Cowan and collaborators have reported an extensive analysis of mouse α-tubulins by isolation of cDNA clones (Lewis *et al.*, 1985a; Villasante *et al.*, 1986). Six functional genes encoding five different polypeptides have been identified by isolation of cloned cDNA sequences from brain and testis libraries. Whether these represent a complete set of mouse α-tubulins is not certain inasmuch as the presence of numerous pseudogenes precludes simple genomic analysis, and the possibility of α-tubulins that are not expressed in the tissues from which the cDNA libraries were constructed cannot be excluded.

In an attempt to identify a complete set of α-tubulins encoded in a vertebrate genome, we have now analyzed α-tubulin genes encoded in the chicken genome. We initially chose this approach for two reasons. First, no evidence for tubulin pseudogenes had been observed for this genome [indeed, unlike mammalian genomes, few chicken pseudogenes for any protein had been documented (Alsip and Konkel, 1986; Robins *et al.*, 1986)]. Second, genomic blots had indicated the presence of only four or five gene segments for α-tubulin (Cleveland *et al.*, 1980). From isolation and analysis of five chicken α-tubulin genes, we now show that three encode functional α-tubulins, one is an unexpected pseudogene and one appears capable of encoding a functional α-tubulin, although we could not demonstrate that it is actually expressed. Combined with two α-tubulins identified by cDNA cloning (Valenzuela *et al.*, 1981; Pratt *et al.*, 1987), a picture for the chicken α-tubulin gene family emerges in which at least five functional genes encode five

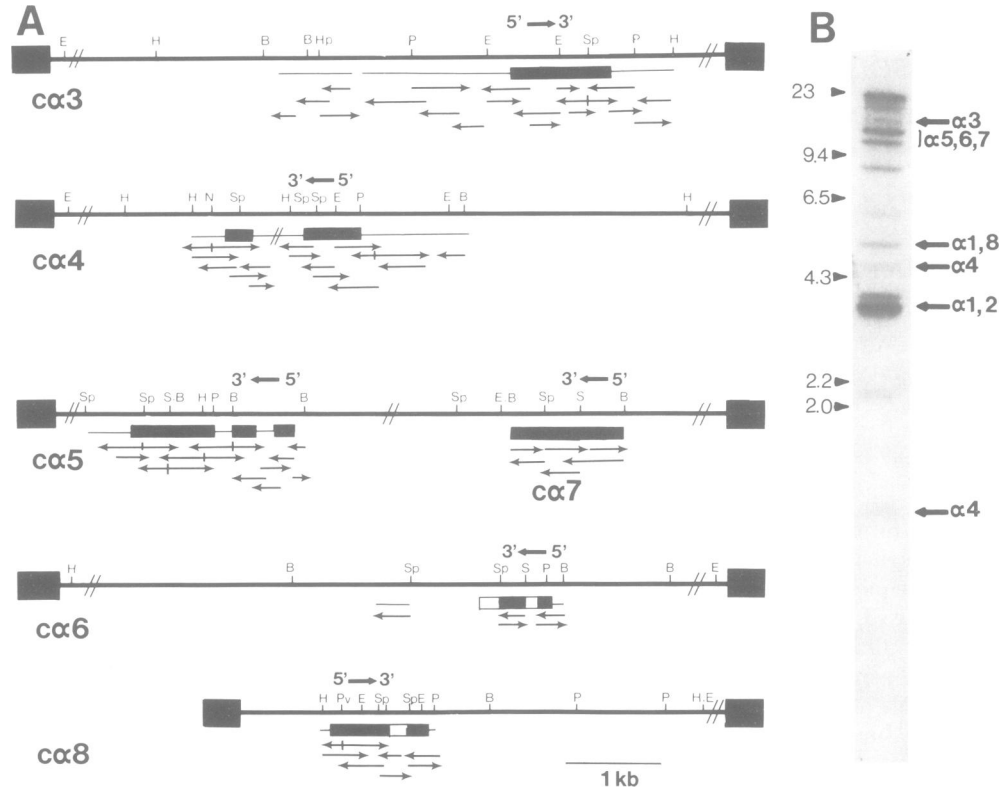


Fig. 1. Characterization of six α-tubulin gene segments. **(A)** Schematic diagrams of genes cα3−cα8. Partial restriction maps of each of five recombinant Charon 30 phages carrying the cα3−cα8 genes are shown. The thick black lines beneath each map represent the coding regions of the corresponding α-tubulin gene segment, whereas the thin lines denote the regions from which doubled stranded DNA sequence was obtained. The arrows at the botton of each gene display the sequencing strategy with which the sequence was determined. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; P, *Pst*I; Sp, *Sph*I; S, *Sal*I; N, *Nde*I, Pv, *Pvu*II. **(B)** The chicken α-tubulin gene family visualized on a blot of genomic DNA digested with *Hin*dIII. Genomic chicken DNA (5 μg) was digested with *Hin*dIII, blotted as in Materials and methods and hybridized to a ^{32}P-labeled probe corresponding to the coding sequences from cα1. Parallel blots were probed with labeled probes prepared from the subcloned genomic fragments of cα3−cα8 (see Materials and methods). Resultant assignments of specific fragments containing α-tubulin sequence homology to individual genes is shown at the right. Sizes markers in kb are shown at the left.

distinct polypeptide isotypes. These isotypes are remarkably divergent particularly in their carboxy-terminal domains, but unlike the β-tubulin family these do not fall into obvious classes that have been conserved among vertebrates.

Results

Isolation and characterization of λ bacteriophage carrying six α-tubulin gene segments

Previously, blot analysis of genomic DNA has been used to demonstrate the presence in the chicken genome of at least four or five segments that appeared to contain complete α-tubulin genes (Cleveland *et al.*, 1980, 1981). To analyze this gene family in detail, 1.6×10^6 recombinants from a genomic library constructed in Charon 30 (library complexity 4×10^5) were screened by DNA hybridization for DNA segments homologous to cα1, a cDNA clone carrying most of the coding region of a widely expressed α-tubulin (Pratt, 1987). Of 67 positive phage, 20 were plaque purified and DNA prepared. Preliminary restriction maps revealed that the cloned fragments represented five distinct genomic segments, one of which contained two α-tubulin genes separated by 2 kb. A partial restriction map of five representative phages containing these six α-tubulin segments is shown in Figure 1A. The regions that contained α-tubulin sequence homology (named cα3−cα8) were determined by hybridization to cα1 sequences and were subcloned into plasmid vectors (see Materials and methods). Blot hybridization with probes corresponding to the amino-terminal codons (amino acids 41−133) or the carboxy-terminal codons (amino acids 376−453) established the apparent direction of transcription of each gene and further suggested that all six segments contained complete α-tubulin-coding regions. Hybridization to 3′ untranslated region probes from the cDNA clones of cα1 (Valenzuela *et al.*, 1981) and cα2 (Pratt *et al.*, 1987) demonstrated that none of the genes encoded cα1 or cα2 (not shown).

Blot analysis of genomic DNA hybridized to a probe containing a nearly complete α-tubulin-coding segment revealed 13 fragments that were distinguishable by size in DNA digested with *Hin*dIII (Figure 1B). To ascertain what proportion of the genomic fragments with high sequence homology to α-tubulin were represented in our six genes and in the cα1 and cα2 cDNAs, DNA segments were prepared from each cloned gene/cDNA and used as probes of parallel genomic blots. (Each probe contained not only α-tubulin-coding regions but also adjacent flanking sequences so that preferential hybridization to the parental gene would be observed.) The analysis yielded the assignments shown in Figure 1B (for details see Pratt, 1987). In almost all cases, the size of the authentic genomic fragment(s) was indistinguishable from the corresponding fragment in the clon-

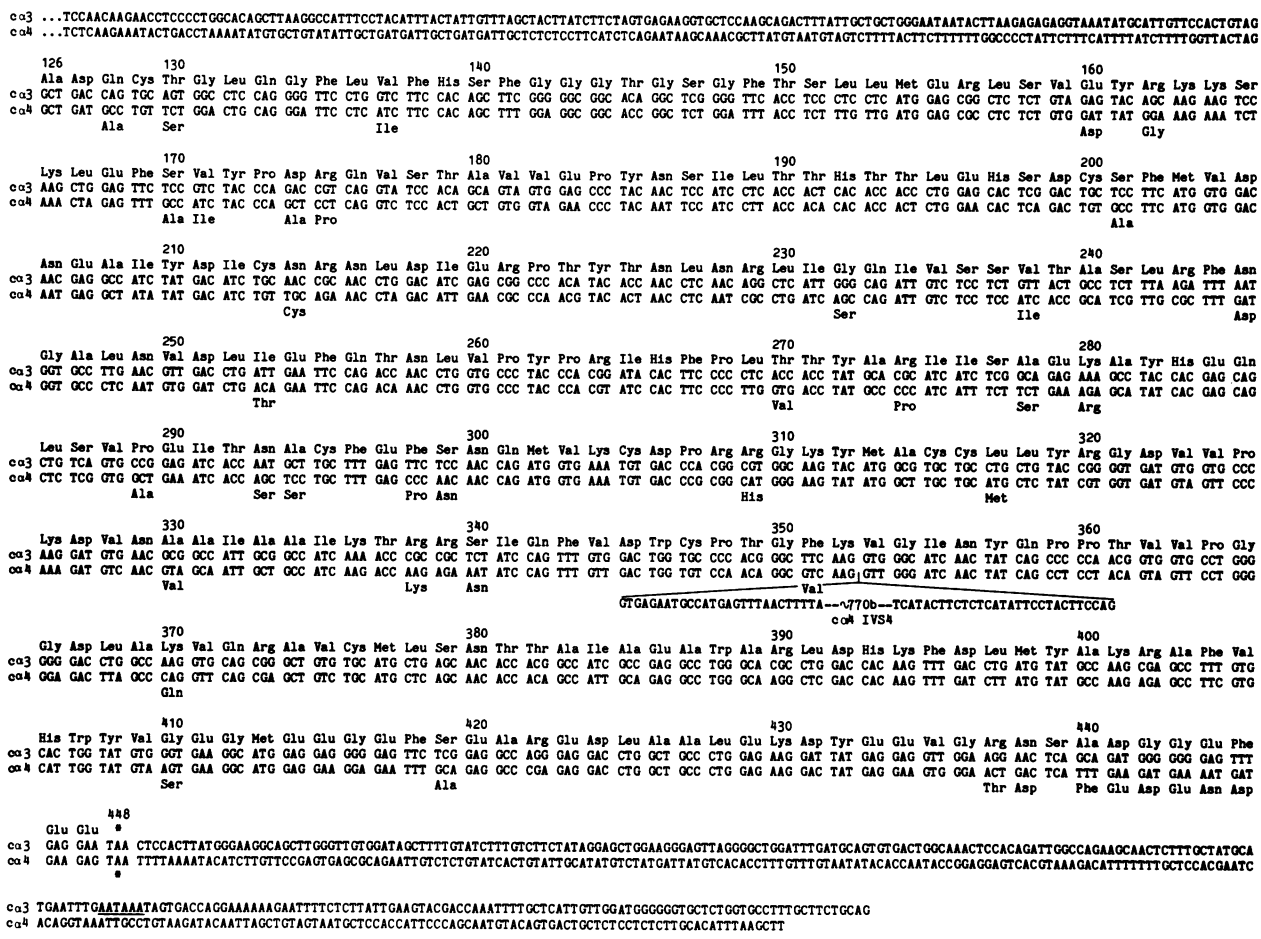


Fig. 2. The DNA and inferred encoded polypeptide sequences for genes cα3 and cα4. The sequence corresponding to a portion of intron 3, followed by all of the coding sequences from codon 126 through to the translation termination codon for both cα3 and cα4 were determined on both strands by the strategies diagrammed in Figure 1A. The asterisk (*) represents the translation termination codon. A putative consensus signal for polyadenylation in the cα3 sequence is underlined. The predicted polypeptide encoded by cα4 is shown only where it differs from cα3. A fourth intervening sequence (~800 bases in length) disrupts the cα4 sequence between codons 352 and 353. The 5′ and 3′ sequences (not shown) of this intron conform to the consensus signals of 5′ and 3′ splice junctions (5′ GTGAGAA . . . and 3′ . . . CCTACTTCCAG).

ed gene, veryifying that the cloned segment did not contain a major cloning artefact. In one instance, however, the map of the cloned cα3 gene predicted that a cα3 *Hin*dIII fragment of ~6 kb should arise on the genomic blot, whereas in fact a much larger fragment is observed (Figure 1B). While this might indicate a cloning artefact, we believe that it is due to restriction site polymorphism in the chicken population because four independent phage-carrying portions of the cα3 gene (each derived from a different cloning event) possess the same structure within the regions of overlap.

We conclude that the six genomic recombinants in conjunction with the cα1 and cα2 cDNA clones appear to contain ~75% of the chicken genomic segments that have high nucleotide sequence homology to cloned α-tubulin cDNAs. Thus, while it is clear that analysis of these eight segments does not comprise the entirety of the chicken α-tubulin gene family, analysis of them should provide insight into the majority of α-tubulin gene complexity in this organism.

Analysis of cα3: an expressed α-tubulin gene without a coded carboxy-terminal tyrosine

The DNA sequence of the region of clone cα3 that is homologous to previously isolated α-tubulin-coding segments was determined according to the strategy diagrammed in Figure 1A. As shown in Figure 2, this yielded an open reading frame of 966 bases that, when translated into protein and compared with the known sequence of other α-tubulins, corresponded to amino acid positions 126 through to the translation termination codon (at position 448). This coding sequence was followed by a putative 3′ untranslated region that contained a consensus sequence for polyadenylation 152 bases 3′ to the translation termination codon. By analogy to the structure of three previously characterized vertebrate α-tubulin genes (in which three introns interrupt the coding sequence at amino acid positions 1/2, 76 and 125/126), we presume that the cα3 sequence corresponds to exon 4 of this gene.

Unfortunately, efforts to locate sequences corresponding to exons 1−3 were unsuccessful. Despite the fact that the cα3 clone contained 9 kb of chicken sequence 5′ to codon 126, hybridization analysis with probes containing the majority of exons 2 and 3 [from either cα1 or from a human α-tubulin cDNA, kα1 (Cowan *et al.*, 1983)] failed to detect the presence of the upstream exons. Since it was still possible that the upstream exons were present but sufficiently divergent to preclude their detection by hybridization, we also searched for the upstream exons by determining the sequence of 2 kb upstream of codon 126 (see Figure 1A). However, no sequences corresponding to those expected for exons 2 and 3 were found. These data indicate that cα3 either

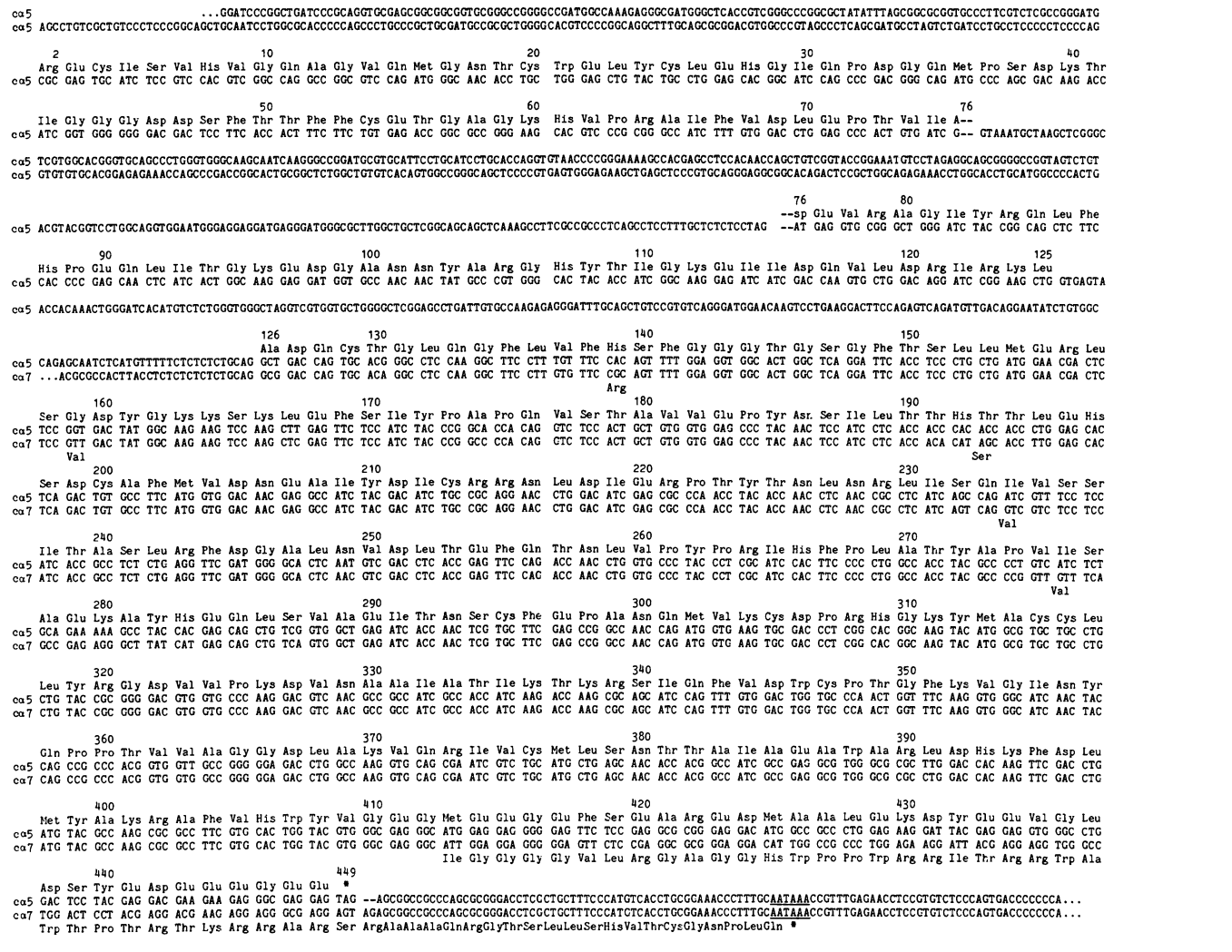


Fig. 3. The DNA and inferred encoded polypeptide sequences for genes cα5 and cα7. The DNA sequences for cα5 and cα7 were determined on both strands according to the strategies diagrammed in Figure 1A. The amino acid sequence for cα5 is shown above the cα5 DNA sequence. Only those amino acid positions that differ from cα5 are shown for cα7. Asterisks (*) represent the translation termination codons. Putative consensus signals for polyadenylation are underlined.

represents a truncated pseudogene in which the 5′ exons have been lost, those exon sequences lie >9 kb upstream, or those exons are very divergent and lie >2 kb upstream.

In any event, determination of the size of the cα3 mRNA using RNA blotting with an oligonucleotide probe complementary to a portion of the putative 3′ untranslated region (see Figure 5A and next section) revealed a doublet of RNAs whose 1.8-kb size is consistent both with the known features of the cα3 gene and with it encoding an authentic α-tubulin polypeptide [e.g. 50-base 5′ untranslated region, 1350-base coding region, 180-base 3′ untranslated region, 100- to 200-base poly(A)tail].

We conclude that cα3 represents a functional, expressed α-tubulin gene and that the 5′ exons must lie >9 kb 5′ to exon 4 or are very divergent and lie >2 kb away.

Comparison of the inferred polypeptide sequence of cα3 with cα1 or with the highly conserved consensus sequence derived from six known mammalian α-tubulins (see Figure 6) revealed that 30 of the 325 residue positions determined (10%) are divergent. The highest concentration of these differences is within the carboxy-terminal region where, compared to cα1, substitutions or deletions have occurred in nine of the last 15 amino acid positions. Of further interest was the absence of an encoded, terminal tyrosine which for many α-tubulin polypeptides undergoes a cyclic post-translational removal and readdition.

DNA sequence analysis of cα4: an α-tubulin gene with an additional intron interrupting codon 352

DNA sequence of the cα4 gene was obtained as shown in the strategy in Figure 1A. The actual sequence is shown along with cα3 in Figure 2. As with the cα3 gene, sequence of all segments with detectable homology to the cα1 coding sequence probe yielded an incomplete gene sequence that began at codon 126 and terminated at the carboxy terminus of α-tubulin. Sequence analysis extending 1 kb upstream did not reveal the presence of the remaining exons. Inspection of the sequence data did reveal, however, that within the sequenced domain an apparent, additional intron segment interrupted the coding sequences between codons 352 and 353. Following this 800-base non-coding sequence, a new fifth exon emerged (in the correct translation frame) from a normal splice junction and continued on to the translation termination codon at position 448. Unlike cα3, no consensus signal for polyadenylation lies within 245 bases 3′ of the translation termination codon.

Like cα3, cα4 displays 10% sequence divergence with the cα1 and mammalian consensus α-tubulins (Figure 6), with the highest degree of divergence localized in the carboxy terminus. Unfortunately, all attempts to demonstrate expression of cα4 were unsuccessful. One such example using RNA blotting with an oligonucleotide complementary to the putative 3′ untranslated region of cα4 failed to detect any RNA transcripts within the 12 tissues and cell lines examined (Figure 5A). Similarly, no cα4 RNAs could be detected in spleen, bursa, thymus or pancreas RNAs (not shown).

Thus, although in the domain sequenced cα4 has retained an open reading frame that could encode an α-tubulin, we are unsure if it is a functional, expressed gene. If it is expressed, either its level of expression is very low or it possesses a restricted range of expression in tissues we have not yet examined.

Analysis of cα5 and cα6: allelic genes encoding a conservative, expressed α-tubulin without an encoded carboxy-terminal tyrosine

The sequence of the cα5 gene (determined by the strategy shown in Figure 1A) is presented in Figure 3. From the 2500 nucleotides of sequence that were determined, we deduced the sequence for 447 encoded amino acids. Like the rat and human α-tubulin genes previously analyzed, cα5 is interrupted by three introns at positions between codons 1 and 2, within codon 76 and between codons 125 and 126. (Since exon 1 encodes only the initiator methionine, it is not possible to identify where in the 5′ sequences this codon and the adjacent 5′ untranslated region are located.) The relatively short introns 2 and 3 (442 and 211 bases respectively) are flanked by appropriate consensus splicing sequences. There are no lesions (translation termination codons etc.) that would suggest that cα5 is other than a functional gene.

Partial DNA sequence analysis of the cα6 gene was also performed according to the strategy shown in Figure 1A. However, comparison of these sequences with cα5 revealed almost complete identity, including those portions within introns (e.g. intron 2 diverged in only three positions). Although restriction mapping had initially revealed many differences between cα5 and cα6, some clear homologies in the maps could also be observed (see Figure 1A). These observations raised the possibility that cα5 and cα6 represented alleles of the same gene rather than independent genetic loci. To test this, for both genes we sequenced an *Ssp*I−*Bam*HI fragment that lies 3′ to the tubulin homology in both genes (see strategy for cα6—Figure 1A). Both sequences were identical, unambiguously indicating that the flanking domain was highly homologous in the two clones. For this to be the case, either cα5 or cα6 arose by recent gene duplication of a large segment of DNA or cα5 and cα6 represent alleles of the same gene which display numerous restriction site polymorphisms. In either case, we cannot distinguish between the RNAs encoded by the two genes and will refer to the corresponding RNA/protein as cα5.

Of all the genes analyzed, cα5 is most closely related to cα1, both at the nucleotide and protein levels. Although 4% of the amino acids are divergent, most of the divergence is concentrated in exons 2 and 3 and at the carboxy terminus. Just as was the case for cα3 and cα4, an encoded carboxy-terminal tyrosine residue is not found.

Analysis of cα7: a chicken α-tubulin pseudogene

The cα7 gene segment was isolated as a gene linked within 2 kb, 5′ to the cα5 genomic fragment. Both genes are oriented in the same transcriptional direction. Nucleotide sequence of cα7 (obtained by the strategy in Figure 1A) is shown in Figure 3 along with cα5. cα7 shows a very strong nucleotide sequence homology to cα5 beginning 15 nucleotides 5′ to the beginning of exon 4 and continuing into the 3′ untranslated region. However, no homology is detected in sequences further 5′ nor can sequences homologous to exons 2 and 3 be detected by hybridization within ~6 kb upstream. Thus, if the 5′ exons exist they must be very divergent in sequence or lie >6 kb away. Further, at amino acid position 413 and again at 426 an extra thymidine residue has been inserted. In each instance these insertions change the translation frame. As a consequence, the predicted polypeptide terminates with 24 'extra' carboxy-terminal amino acids encoded by sequences that lie within the 3′ untranslated region of cα5. In view of the frameshift lesions and the possible loss of the 5′ exons, we conclude that cα7 is a pseudogene, the apparent product of an unequal crossover event during recombination. [Note that if cα7 were actively transcribed, its RNA could not be distinguished from cα5 by 3′ untranslated region probes. However, since cα5 contains no apparent lesions that would disrupt its expression, we believe that any mRNA detected (see below and Figure 5A) is encoded by cα5.]

Analysis of cα8: a functional gene with an additional intron between codons 352 and 353

cα8 was subjected to DNA sequence analysis (Figure 1A) and the sequences determined are shown in Figure 4. Again, only sequences 3′ to codon 126 were determined [the cα8 gene segment contains only 1 kb of sequences (presumably intron 3) lying 5′ to codon 126]. Immediately apparent from inspection of the figure is that this gene segment also contains an apparent, additional 201-base intron between codons 352 and 353 (the same position as the extra intron in cα4).

Comparison of the predicted polypeptide sequence encoded by cα8 to that of the other genes (Figure 6) reveals that cα8 most closely resembles cα2 and cα4 in the type of amino acid substitutions made, but overall is still more closely related to cα1 (differing in only 4% of positions). Once again, a major cluster of divergence appears in the carboxy-terminal region of the coding sequence, where six substitutions and/or deletions have been accepted in the last 15 amino acid positions. Alone among cα3−cα8, cα8 contains the encoded terminal tyrosine found in most previously analyzed α-tubulins.

Determination of the pattern of expression of cα3−cα8

To determine where cα3−cα8 were expressed in chicken tissues, ^{32}P-labeled probes that would specifically identify the corresponding mRNAs were prepared from the 3′ untranslated regions of cα3, cα4, cα5/6 and cα8. These were used to detect the corresponding RNA transcripts on parallel blots of RNAs isolated from a series of chicken tissues or cell lines. Autoradiograms of the resultant blots are shown in Figure 5A along with an additional blot probed with a coding sequence probe (from cα1) that we believe detects all α-tubulin mRNAs. As expected, α-tubulin mRNAs were detected in all cells and tissues using the coding sequence

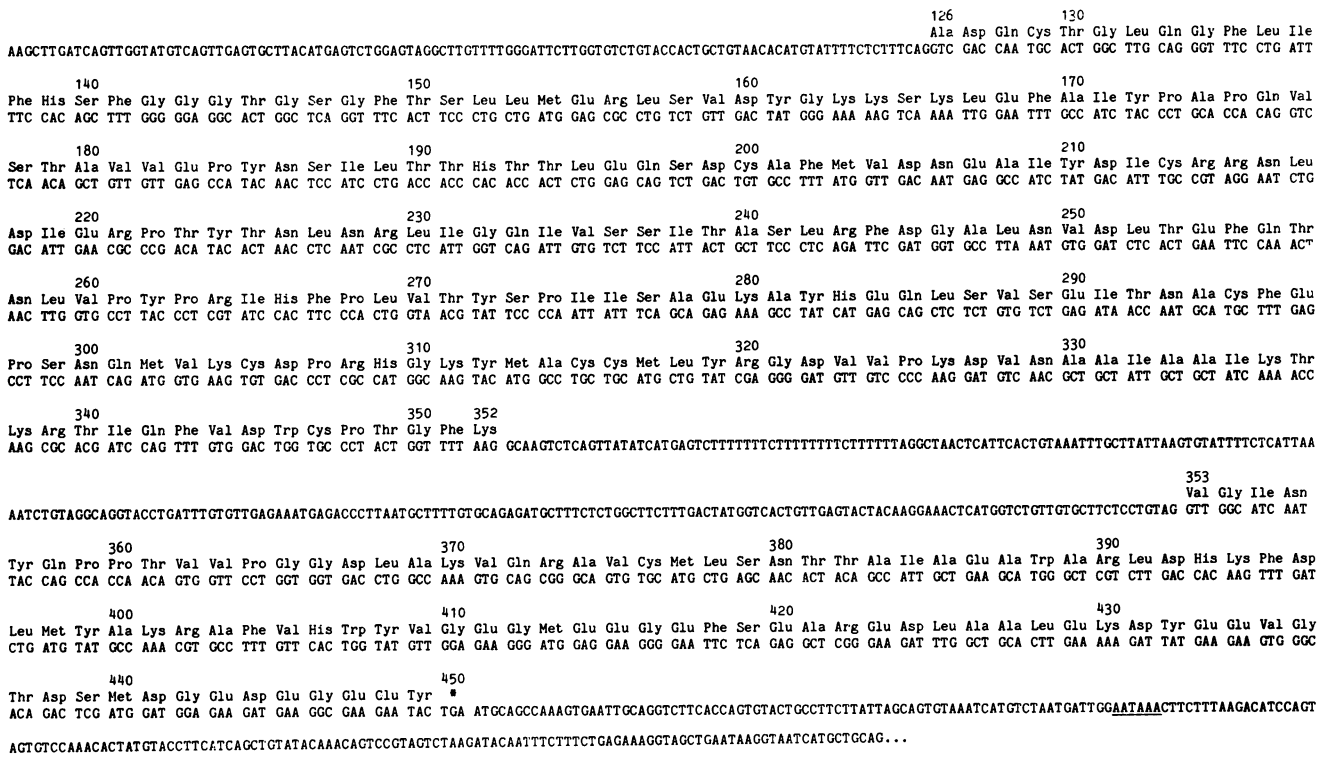


Fig. 4. The DNA and inferred encoded polypeptide sequences for gene cα8. The DNA sequence of cα8 beginning in intron 3 and continuing through the putative site for polyadenylation was determined by the strategy diagrammed in Figure 1A. The corresponding amino acid sequence is shown above the DNA sequence. The asterisk (*) represents the translation termination codon. The putative consensus sequence for polyadenylation is underlined.

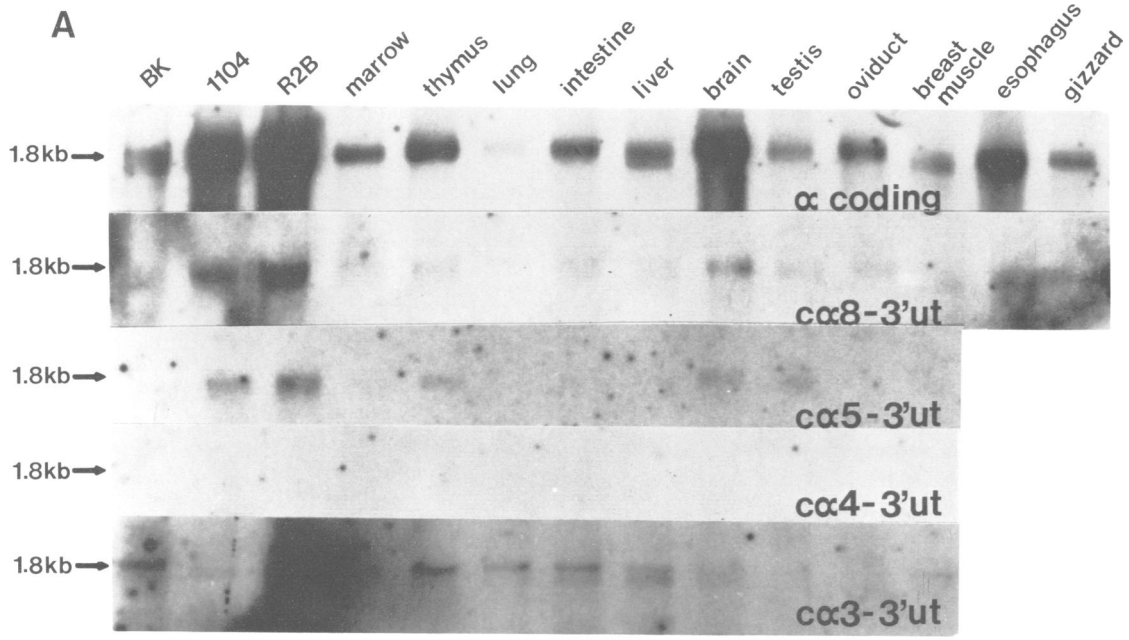
probe. A distinctly different pattern was seen for cα3. For this gene, a ^{32}P-labeled oligonucleotide complementary to a portion of the putative 3′ untranslated region identified a doublet of RNAs of ~1.8 kb in length in RNAs derived from several tissues, most prominently intestine and liver. (We cannot distinguish among the possibilities that could yield RNAs of slightly different size.) On the other hand, no RNAs could be detected with an oligonucleotide probe complementary to the cα4 3′ untranslated region, either in the 12 samples analyzed in Figure 5A or in spleen, bursa, thymus or pancreas (not shown). Thus, despite the fact that the cα4 gene contains no lesions that would suggest it to be other than a functional gene, we could not document that it is actually expressed. For cα5, a relatively high level of expression was found in two of three lymphocyte lines and in thymus. Low levels of expression were found in brain and testis, the only other cells or tissues in which cα5 transcripts were detected. Lastly, cα8 RNAs were detected ubiquitously at low levels in all 14 tissues surveyed, and at substantially higher levels in brain and in two of three lymphocyte lines. A summary of these hybridization results is presented in Figure 5B.

Discussion

In conjunction with two previously analyzed chicken α-tubulin cDNAs, the characterization of the cα3−cα8 genes has provided a survey of the α-tubulin gene family in one vertebrate genome. cα1−cα8 appear to account for ~75% of the genomic α-tubulin segments. Within this family of sequences, five genes appear encode functional tubulins (cα1, cα2, cα3, cα5 and cα8), whereas one is almost certainly a pseudogene (cα7) and one (cα4) may yet be a functional gene (although we were unable to document its expression). Potentially, several more functional α-tubulins may be encoded by genes not yet isolated.

Detailed comparison of the polypeptides encoded by the genes now identified to be functional is presented in Figure 6. (Also included is cα4 since it may be functional.) Only one (cα1) is highly homologous in amino acid sequence to the consensus found in most previously sequenced vertebrate α-tubulins (Figure 6). For the portions of the sequences that have been determined, the remaining four (cα2, cα3, cα5 and cα8) diverge from the consensus sequence in 17, 10, 4 and 4% of residue positions. Most striking is that all but cα1 show substantial sequence heterogeneity in the carboxy-terminal positions beyond residue 435, where substitutions and deletions have been accepted in 100% of the positions (these data are summarized in Figure 7). This is analogous to the situation for β-tubulin where the major differences among β-tubulin isotypes are within the carboxy-terminal 15 residues (e.g. Sullivan and Cleveland, 1986).

Analysis of the chicken α-tubulins represents the second detailed look at a vertebrate α-tubulin family. Previously, Cowan and collaborators (Villasante *et al.*, 1986) identified six different mouse α-tubulin cDNAs and showed them to encode five different polypeptides. These included a polypeptide ubiquitously expressed, a sister gene whose expression is a subset of the first, two genes expressed only in testis, and one gene expressed at low levels in many tissues and which yields only an aberrant transcript in brain and testis. Although there are some similarities, the patterns of expression of the five functional chicken α-tubulins (summarized in Figure 5B) do not obviously parallel those in mouse. Fur-



B

cα1	Ubiquitous, brain
cα2	Testis-specific
cα3	Ubiquitous at low levels; liver,intestine,marrow, BK lymphocyte cell line
cα4	?
cα5/6	Brain, testis, thymus, R2B and 1104 lymphocyte cell lines
cα8	Ubiquitous at low levels; brain, R2B and 1104 lymphocyte cell lines

Fig. 5. RNA blot analysis of the expression of genes cα1−cα8 in chicken tissues and cell lines. (**A**) Equal amounts of poly(A)$^+$ RNA from various chicken tissues and cell lines were electrophoresed under denaturing conditions on five parallel gels and blotted to nitrocellulose. Filters were hybridized with a coding sequence probe from cα1 that is believed to hybridize efficiently to all α-tubulin mRNAs (top panel) or with probes (see Materials and methods) that specifically recognize cα8, cα5, cα4 or cα3 RNAs respectively. BK, 1104 and R2B are chicken lymphocyte cell lines. (**B**) Summary of the patterns of expression of cα1−cα8 as deduced from RNA blots. Data for expression of cα1 and cα2 are from Pratt (1987) and Pratt *et al.* (1987) respectively.

ther, the divergence in sequence among chicken α-tubulins is substantially greater than the 4% maximum divergence found thus far in mouse.

Consideration of the heterogeneity within at least the chicken α-tubulin carboxy-terminal sequences suggests the possibility that the polypeptides may be functionally distinguishable. Only cα1 and cα8 contain encoded terminal tyrosine residues, whereas all but one α-tubulin previously analyzed in vertebrates (Mα4—Villasante *et al.*, 1986) has contained an encoded carboxy-terminal tyrosine. In many tissues α-tubulins are known to undergo a cyclic process of detyrosination (through the action of a carboxypeptidase) followed by re-tyrosination (by a specific α-tubulin−tyrosine ligase). Further, a subset of microtubules in cultured cells that is enriched in detyrosinated tubulin are also more stable (Kreis, 1987; Wehland and Weber, 1987), although detyrosination is probably not the cause of the increased stability (Khawaja *et al.*, 1988).

Despite this, the determinants that specify α-tubulin as the unique substrate for the tyrosine ligase have not been identified. However, the divergence of the carboxy-terminal residues among the chicken polypeptides clearly suggests the possibility that one or more of these α-tubulins does not participate in this cycle. Since it has been demonstrated (Gundersen *et al.*, 1987; Wehland and Weber, 1987) that (at least for the consensus α-tubulins such as cα1) it is the tyrosinated subunit that is the major subunit used for assembly and that detyrosination occurs post-polymerization, carboxy-terminal sequence heterogeneity could easily produce functional distinctions among α-tubulin isotypes.

So what can we conclude concerning the significance of the α-tubulin multigene family? Unlike the case for β-tubulin where evolutionarily conserved polypeptide isotypes have been identified both by their unique carboxy-terminal sequences and programmes of expression, comparison of the carboxy-terminal sequences from all available vertebrate α-tubulins (compared in Figure 7) provides little support for the existence of conserved isotypic classes of α-tubulin polypeptides. (In fact, except for cα1, no other chicken isotype corresponds in sequence to any of the remaining four mouse α-tubulins.) Nor does consideration of the pattern of expression offer firm support for such a hypothesis. The

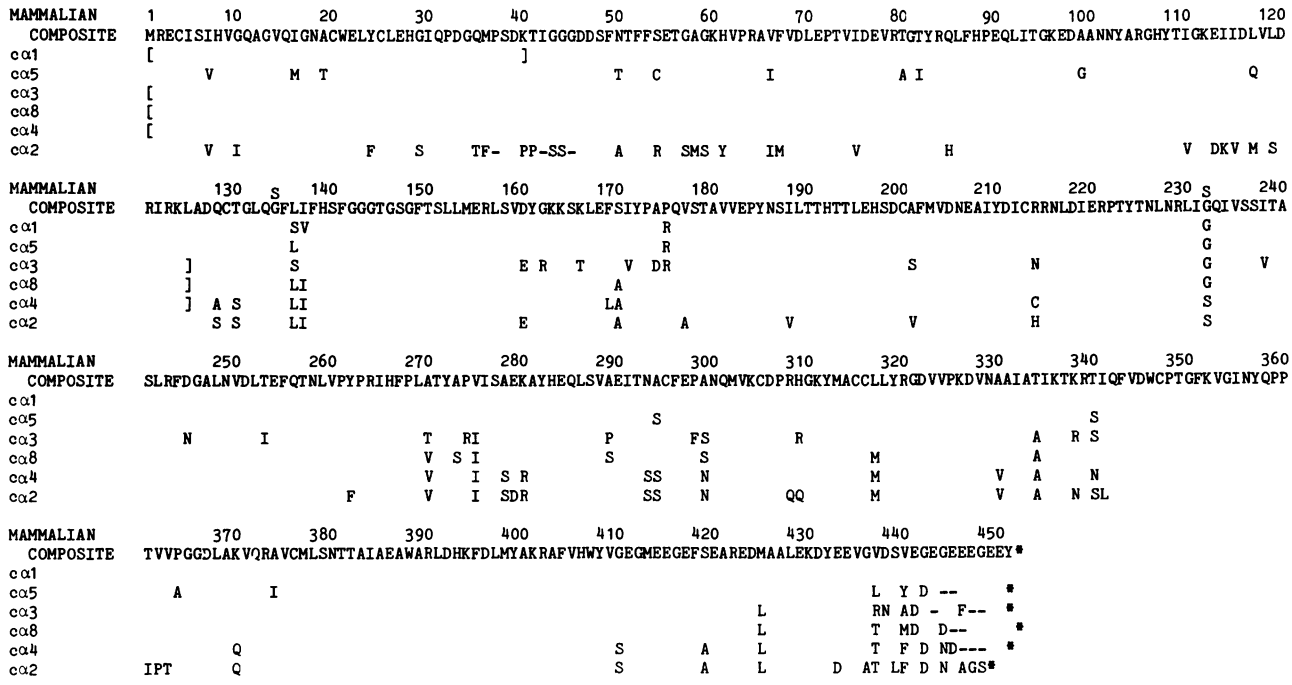


Fig. 6. Comparison of the polypeptide sequences of the known chicken α-tubulins. The one-letter amino acid code is utilized to display the sequences of cα1−cα8. A composite mammalian sequence [represented by human genes bα1 and kα1 (Cowan *et al.*, 1983), a porcine sequence (Ponstingl *et al.*, 1981), a rat α-tubulin (Lemischka *et al.*, 1981) and two Chinese hamster α-tubulins (Elliott *et al.*, 1986)] is displayed on the top line. The cα1−cα8 sequences are shown beneath the composite, explicitly showing only the residue positions that differ with the composite sequence. Dashes (—) represent deletions introduced to maintain homology. Translation termination codons are denoted by (*). Portions of sequence that have not yet been determined (the amino-terminal 125 amino acids of cα3, cα4 and cα8 and the amino-terminal 39 residues of cα1) are denoted on the figure by the areas contained within brackets.

absence of such evidence may simply be the result of failure to isolate the homologous sequences from multiple species. Alternatively, it may indicate that the carboxy-terminal sequences of α-tubulin can tolerate substantial sequence divergence without disruption of function as has been demonstrated for an amino-terminal variable domain between residues 30 and 50 (Schatz *et al.*, 1987). Although the evolutionarily conserved cyclic process of carboxy-terminal tyrosination/detyrosination argues against this latter possibility, additional analyses will be necessary to settle this point. What is clear is that significantly more sequence heterogeneity is present in vertebrate α-tubulins than had previously been recognized.

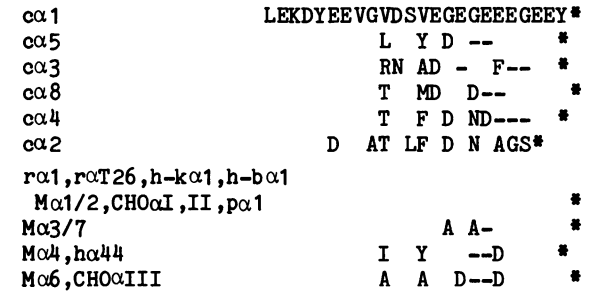


Fig. 7. Summary of carboxy-terminal sequences of chicken cα1−cα8 tubulins. The carboxy-terminal sequences for all known vertebrate α-tubulins are aligned. Asterisks (*) represent the translation termination codons. Dashes (—) represent deletions introduced so as to maintain homology. rα1 and rαT26 are taken from Lemischka *et al.* (1981) and Ginzburg *et al.* (1981) respectively; h-kα1 and h-bα1 are from Cowan *et al.* (1983); CHO I, II and III are from Elliott *et al.* (1986); pα1 is from Ponstingl *et al.* (1981); Mα1−Mα7 are from Villasante *et al.* (1986); hα44 is from Dobner *et al.* (1987).

Materials and methods

Genomic library screening

A genomic library of embryonic chicken DNA was obtained from Dr Bjorn Vennstrom (EMBL, Heidelberg—Vennstrom and Bishop, 1982). The library was constructed by partial digestion of chicken DNA with *Mbo*I and selection of DNA fragments of ~15−20 kb in length. These were then ligated into Charon 30 which had been digested with *Bam*HI. Approximately 1.6 × 10^6 phage (four times the complexity of the library) were screened for α-tubulin sequences by the protocol of Maniatis *et al.* (1978). A ^{32}P-labeled probe was prepared from an embryonic chick brain cDNA clone originally called pT1, renamed cα1 (Pratt *et al.*, 1987), that contains the coding sequence for α-tubulin beginning at amino acid 42 and continuing through the 3′ complete untranslated region (Valenzuela *et al.*, 1981). The probe was prepared by the random priming method of Shank *et al.* (1978). Conditions for hybridization and washing were as given below for DNA blots.

Restriction maps of the cloned genomic DNA were prepared using restriction endonucleases according to suppliers' recommendations.

Gel electrophoresis and blotting

Gel electrophoresis of DNA was performed on vertical slab gels of 0.8% agarose containing 40 mM Tris−HCl, pH 8.1, 20 mM sodium acetate, and 2 mM EDTA. Gels were stained with 1 μg/ml ethidium bromide to visualize the DNA bands. DNA was transferred to nitrocellulose filters according to the method of Southern (1975).

Gel electrophoresis of RNA was carried out on vertical slab gels cast from 1.0% agarose containing 2.2 M formaldehyde (Boedtker, 1971). RNA was transferred to nitrocellulose by the method of Thomas (1980).

Hybridization conditions for DNA and RNA blots

DNA and RNA blots were prehybridized for 2−16 h at 41°C in 50% formamide, 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate), 20 mM Hepes (pH 7.4), 0.4 mg/ml yeast tRNA, 0.06 mg/ml sonicated, denatured herring sperm DNA, 0.06% bovine serum albumin, 0.06% Ficoll, 0.06% polyvinylpyrrolidone. The filters were then hybridized in the same solution containing 10^6 d.p.m./ml of a ^{32}P-labeled probe that had been denatured by boiling for 3 min. These filters were washed in three changes of 0.1 × SSC, 0.1% SDS at 51°C for a total wash time of 1 h.

Hybridization conditions for DNA and RNA blots probed with oligonucleotides

Filters were prehybridized for 24 h in 20% formamide, 5 × SSC, 20 mM phosphate buffer (pH 6.5) and 0.06% albumin, 0.06% Ficoll and 0.06% polyvinylpyrrolidone. The blots were then hybridized in the same solution containing 10^6 d.p.m./ml of the appropriate ^{32}P-labeled probe (labeled with polynucleotide kinase and [γ-^{32}P]ATP) for a minimum of 24 h. The blots were washed to a final stringency of 2 × SSC at 60°C according to Villasante *et al.* (1986).

Subcloning isolated chicken α-tubulin genes

Regions of phage DNA containing each individual α-tubulin hybridizing region were subcloned in pBR322 as follows.

pcα3: a 6.0 kb genomic *Hin*dIII fragment was subcloned into the *Hin*dIII site of pBR322, such that the direction of transcription of the α-tubulin segment would be in the clockwise direction in the normal presentation of pBR322.

pcα4: a 5.0-kb *Bam*HI fragment was subcloned into the *Bam*HI site of pBR322 in the counterclockwise orientation to the pBR322 vector.

pcα5: a 9.4-kb *Eco*RI fragment was subcloned into the *Eco*RI site of pBR322 in the counterclockwise orientation to the pBR322 vector.

pcα7: a 5.0-kb *Eco*RI fragment was subcloned into the *Eco*RI site of pBR322 in the counterclockwise orientation to the pBR322 vector.

pcα6: a 12-kb fragment bounded by *Eco*RI and *Hin*dIII was subcloned into double-cut pBR322.

pcα8: a 6-kb *Hin*dIII fragment was subcloned into the *Hin*dIII site of pBR322 in the clockwise orientation.

Construction of DNA probes that hybridized with specific α-tubulin genes

cα3: an antisense oligonucleotide probe of 29 bases complementary to the 3′ non-coding region directly proximal to the translation stop codon was used as probe. The probe was 5′ end-labeled with [γ-^{32}P]dATP and polynucleotide kinase.

cα4: an antisense oligonucleotide probe of 29 bases complementary to nucleotides 20–49 3′ to the translation stop codon (with 1 taken as the first base 3′ to the stop codon) was synthesized for use as a probe and end-labeled as for cα3.

cα5: a region of DNA from an *Ava*II site 18 nucleotides downstream of the stop codon to an *Ava*II site ~200 nucleotides more 3′ to the gene was used as a probe.

cα8: a region of DNA from a *Taq*I site at the 3′ end of the coding region (30 bases upstream from the translation termination codon) extending to the distal *Pst*I site 209 nucleotides downstream was used as a probe.

We confirmed that the probes were specific to the corresponding gene/RNA by hybridization of the probes to (i) a panel of blots containing all of the cloned sequences and (ii) to genomic blots of chicken DNA (not shown).

DNA sequence analysis

Three methods of obtaining M13 clones carrying appropriate α-tubulin gene segments were utilized for DNA sequence analysis. First, restriction fragments were directly subcloned into the replicative form of M13 mp18 or mp19. Second, progressive deletion series were obtained using *Bal*31 exonuclease as described in detail by Lewis *et al.* (1985a). These deletions were ligated into appropriately digested M13 with T4 DNA ligase under conditions recommended by the supplier. Third, the kilo-sequencing method of Barnes *et al.* (1983) was used following initial subcloning of specific gene fragments into M13. Briefly, the replicative form of the phage was nicked in a random position using DNase I cleavage in the presence of ethidium bromide. The nick was extended into a gap with exonuclease III and *Bal*31 nuclease was used to make a double-stranded break at the site. A restriction site proximal to the M13 sequencing priming site was then digested to release the region between the two sites and the remaining portion was religated together under conditions encouraging self-religation. Phage-carrying deletions were then sized on agarose slab gels to select an appropriate series of deletion clones.

DNA sequence was obtained from single-stranded M13 templates using the dideoxy method of Sanger *et al.* (1977). Sequencing reactions were loaded onto 9 M urea–6% acrylamide sequencing gels and run for 2 and/or 5 h. The sequence was visualized by autoradiography.

Preparation of RNA

Total RNA was prepared from tissues and cells by the method of Chirgwin *et al.* (1979). Briefly, cells or frozen tissues were disrupted in 6 M guanidine thiocyanate. Debris was pelleted by low-speed centrifugation and the supernatant layered on a cushion of cesium chloride. After centrifugation, the RNA pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated water and stored at −80°C. Poly(A)$^+$ RNA was isolated from total RNA by passage over a column of oligo(dT) cellulose (Type III, Collaborative Research). Poly(A)$^+$ RNA was precipitated with ethanol, resuspended in distilled water and stored at −80°C.

Acknowledgements

We thank Dr Bjorn Vennstrom for providing the chicken genomic library. This work has been supported by a grant from the NIH to D.W.C., who is also the recipient of an NIH Research Career Development Award.

References

Alsip,G.R. and Konkel,D.A. (1986) *Nucleic Acids Res.*, **14**, 2123–2138.
Barnes,W.M., Bevan,M. and Son,P.H. (1983) *Methods Enzymol.*, **101**, 98–122.
Boedtker,H. (1971) *Biochem. Biophys. Acta*, **240**, 448–453.
Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J.. and Rutter,W.J. (1979) *Biochemistry*, **18**, 5294–5299.
Cleveland,D.W. (1987) *J. Cell Biol.*, **103**, 381–383.
Cleveland,D.W., Lopata,M.A., Cowan,N.J., MacDonald,R.J., Rutter,W.J. and Kirschner,M.W. (1980) *Cell*, **20**, 95–105.
Cleveland,D.W., Hughes,S.H., Stubblefield,E., Kirschner,M.W. and Varmus,H.E. (1981) *J. Biol. Chem.*, **256**, 3130–3134; *Cell*, **25**, 537–546.
Cleveland,D.W. and Sullivan,K.F. (1985) *Annu. Rev. Biochem.*, **54**, 331–365.
Cowan,N.J., Dobner,P.R., Fuchs,E.V. and Cleveland,D.W. (1983) *Mol. Cell. Biol.*, **3**, 1738–1745.
Dobner,P.R., Kislauskis,E., Wentworth,B.M. and Villa-Komaroff,L. (1987) *Nucleic Acids Res.*, **15**, 199–218.
Elliott,E.M., Henderson,G., Sarangi,F. and Ling,V. (1986) *Mol. Cell. Biol.*, **6**, 906–913.
Gibbons,I.R. (1975) In Inove,S. and Stephens,R.E. (eds), *Molecules and Cell Movement*. Raven Press, New York, pp. 207–232.
Ginzburg,I., Behar,L., Givol,D. and Littauer,U.Z. (1981) *Nucleic Acids Res.*, **9**, 2691–2697.
Gundersen,G.G., Khawaja,S. and Bulinski,J.C. (1987) *J. Cell Biol.*, **105**, 251–264.
Hall,J.L. and Cowan,N.J. (1985) *Nucleic Acids Res.*, **13**, 207–222.
Hall,J.L., Dudley,L., Dobner,P.R., Lewis,S.A. and Cowan,N.J. (1983) *Mol. Cell. Biol.*, **3**, 854–862.
Hayden,J.H. and Allen,R.D. (1984) *J. Cell Biol.*, **99**, 1785–1793.
Heuser,J. and Kirschner,M.W. (1980) *J. Cell Biol.*, **86**, 212–234.
Khawaja,S., Gundersen,G.G. and Bulinski,J.C. (1988) *J. Cell Biol.*, in press.
Kreis,T.E. (1987) *EMBO J.*, **6**, 2597–2606.
Lee,M.G.-S., Lewis,S.A., Wilde,C.D. and Cowan,N.J. (1983) *Cell*, **33**, 477–487.
Lemischka,I.R., Farmer,S.R., Racaniello,V.R. and Sharp,P.A. (1981) *J. Mol. Biol.*, **150**, 101–120.
Lemischka,I.R. and Sharp,P.A. (1982) *Nature*, **300**, 330–335.
Lewis,S.A., Lee,M.G.-S. and Cowan,N.J. (1985a) *J. Cell Biol.*, **101**, 852–861.
Lewis,S.A., Gilmartin,M.E., Hall,J.L. and Cowan,N.J. (1985b) *J. Mol. Biol.*, **181**, 11–20.
Lopata,M.A. and Cleveland,D.W. (1987) *J. Cell Biol.*, **105**, 1707–1720.
Maniatis,T., Hardison,R.C., Lacey,E., Lauer,J., O'Connell,C., Quon,D., Sim,G.K. and Efstratiadis,A. (1978) *Cell*, **15**, 687–701.
Montiero,M.J. and Cleveland,D.W. (1988) *J. Mol. Biol.*, **199**, 439–446.
Murphy,D.B., Wallis,K.T., Machlin,P.S., Ratrie,H. and Cleveland,D.W. (1987) *J. Biol. Chem.*, **262**, 14305–14312.
Ponstingl,H., Krauhs,E., Little,M. and Kempf,T. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2757–2761.
Pratt,L. (1987) Ph.D. thesis, The Johns Hopkins University.
Pratt,L. Okamura,S. and Cleveland,D.W. (1987) *Mol. Cell. Biol.*, **7**, 552–555.
Robins,A.J., Wang,S.W., Smith,T.F. and Wells,J.R. (1986) *J. Biol. Chem.*, **261**, 18–20.
Sanger,F., Nicklen,S. and Coulsen,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Schatz,P., Georges,G., Solomon,F. and Botstein,D. (1987) *Mol. Cell. Biol.*, **7**, 3799–3805.
Shank,P.R., Hughes,S.H., Kung,H.-J., Majors,J.E., Quintrell,N., Guntaka,R.V., Bishop,J.M. and Varmus,H.E. (1978) *Cell*, **15**, 1383–1395.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.

Sullivan,K.F. and Cleveland,D.W. (1984) *J. Cell Biol.*, **99**, 1754–1760.
Sullivan,K.F. and Cleveland,D.W. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4327–4331.
Sullivan,K.F., Lau,J.T.Y. and Cleveland,D.W. (1985) *Mol. Cell. Biol.*, **5**, 2454–2465.
Sullivan,K.F., Havercroft,J.C., Machlin,P.S. and Cleveland,D.W. (1986a) *Mol. Cell. Biol.*, **6**, 4409–4418.
Sullivan,K.F., Machlin,P.S., Ratrie,H.,III and Cleveland,D.W. (1986b) *J. Biol. Chem.*, **261**, 13317–13322.
Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205.
Vale,R.D., Schnapp,G.J., Reese,T.S. and Sheetz,M.P. (1985) *Cell*, **40**, 449–454.
Valenzuela,P., Quiroga,M., Zaldivar,J., Rutter,W.J., Kirschner,M.W. and Cleveland,D.W. (1981) *Nature*, **289**, 650–655.
Vennstrom,B. and Bishop,J.M. (1982) *Cell*, **28**, 135–143.
Villasante,A., Wang,D., Dobner,P.R., Dolph,P., Lewis,S.A. and Cowan,N.J. (1986) *Mol. Cell. Biol.*, **6**, 2409–2419.
Wang,D., Villasante,A., Lewis,S.A. and Cowan,N.J. (1986) *J. Cell Biol.*, **103**, 1903–1910.
Wehland,J. and Weber,K. (1987) *J. Cell Sci.*, **88**, 185–203.
Wilde,C.D. Crowther,C.E., Cripe,T.D., Lee,M.G. and Cowan,N.J. (1982) *Nature*, **297**, 83–84.

Received on November 3, 1987; revised on January 22, 1988