Molecular Cloning and Functional Characterization of Chicken Brain Tau: Isoforms with up to Five Tandem Repeats†

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ABSTRACT: Tau is a major microtubule-associated protein in mammalian brain, where it exists as multiple isoforms that are produced from a single gene by alternative mRNA splicing. Here we present the first report on the structure and function of tau protein from a nonmammalian vertebrate. In the adult chicken brain, five main tau isoforms are expressed. One isoform has three tandem repeats, two isoforms have four repeats each, and two isoforms have five repeats each. Similar to mammalian tau, some chicken tau isoforms contain an amino-terminal insert of 53 amino acids. Unlike mammalian tau, a 34 amino acid insert in the proline-rich region upstream of the repeats is alternatively spliced in chicken tau. It is preceded by a constitutively expressed sequence of 17 amino acids that is absent in tau from human and rodent brains. The expression of chicken tau isoforms and their phosphorylation are developmentally regulated, similar to what has been described in mammalian brain. Functionally, chicken tau isoforms with five repeats have the greatest ability to promote microtubule assembly, followed by isoforms with four and three repeats, respectively. The 34 amino acid insert positively influences both the rate and the extent of microtubule assembly, whereas the 53 amino acid insert only influences the extent of assembly.

Microtubules are essential cytoskeletal components made of repeating $\alpha\beta$-tubulin heterodimers that are labile unless stabilized by other molecules (*1*). Accordingly, most cells express microtubule-associated proteins (MAPs) which stabilize microtubules. Of the neuronal MAPs, tau protein is one of the most widely studied, largely because of its role in the etiology and pathogenesis of a number of neurodegenerative diseases (*2*).

Six tau isoforms are produced in adult human brain by alternative mRNA splicing from a single gene (*3*−*6*). They range from 352 to 441 amino acids in length and differ from each other by the presence or absence of 29 or 58 amino acid inserts located in the N-terminal half and an additional 31 amino acid repeat located in the C-terminal half. Inclusion of the latter produces the three isoforms with four tandem repeats each; the other three isoforms have three repeats each. The repeats constitute the microtubule-binding domains of tau (*7*, *8*), with four-repeat tau being better at promoting microtubule assembly and at binding to microtubules than three-repeat tau (*9*, *10*). Similar levels of three-repeat and four-repeat tau isoforms are expressed in the adult human cerebral cortex (*9*). The expression of tau protein isoforms is developmentally regulated. Thus, in immature human brain, only the shortest isoform (three repeats and no N-terminal inserts) is expressed (*5*). Mutations in the tau gene cause the inherited disease frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)[1] (*2*, *11*−*13*). Functionally, many mutations reduce the ability of tau to promote microtubule assembly. Moreover, an imbalance in the ratio of three-repeat to four-repeat tau that results in the relative overproduction of tau with four repeats leads to FTDP-17. This indicates that a tight regulation of tau isoform ratios is essential for preventing neurodegeneration and dementia.

The tau repeats are conserved from nematodes to humans and are 60−70% homologous to the repeats of the high-molecular weight neuronal MAP2 and the more widely expressed MAP4 (*14*). In all vertebrates examined, tau is subject to a similar developmentally regulated alternative mRNA splicing. In adult brain, however, the number of expressed tau isoforms varies between species. Thus, rodents only express three isoforms, each with four tandem repeats (*15*). In other species, three-repeat isoforms are also expressed alongside tau isoforms with four repeats (*16*). *Caenorhabditis elegans* and *Drosophila melanogaster* express only one protein with tau-like repeats (*14*, *17*, *18*). Unlike mammalian tau, the invertebrate proteins contain up to five tandem repeats.

Here we have characterized chicken tau at the molecular level. As in other vertebrates, one three-repeat isoform is present early in development. However, in adult chicken brain, tau isoforms with three, four, and five repeats are expressed. Functionally, five-repeat tau was best at promoting microtubule assembly, followed by tau proteins with four and three repeats, respectively.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid: EGTA, ethylene-glycol-bis-(2 aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17, PIPES, piperazine-NN′-bis-2-diethanesulfonic acid; PTL, protein tau-like; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase polymerase chain reaction; TLP, tau-like protein; XTP, *Xenopus laevis* tau-like protein.

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EXPERIMENTAL PROCEDURES

Isolation of cDNA Clones Encoding Chicken Brain Tau. A 450 bp PCR fragment from exons 9−11 of rat tau was labeled by random priming and used to screen a chicken brain cDNA library in lambda gt10 (Clontech) at high stringency. Six positive clones were isolated from 5×10^5 plaques. Purified λ DNAs were digested with EcoRI and BamHI, the inserts subcloned into M13mp18 and sequenced using the dideoxy chain termination method with synthetic oligonucleotides as primers. Alternatively, the inserts were subcloned into the plasmid vectors pSG5 (Stratagene) or pcDNA3.1 (Invitrogen) prior to DNA sequencing.

RT-PCR Analysis. Total RNA was extracted using the Trizol reagent (Life Technologies) from 250 mg chicken brain dissected from 8, 10, 15, and 19 day-old embryos, as well as from newborn and adult animals. To prepare cDNA, 2 μg of total RNA was annealed with 20 pmol of the antisense primer 5′-CTCTAGAATTCCCCCCTCATTCTC-TC-3′ in a 12 μL reaction mixture. Reverse transcription used a 30 min incubation with SuperScript II reverse transcriptase (20 U/μL, Life Technologies) at 42 °C, followed by a 10 min incubation with RNase H at 55 °C. PCR amplification (denaturation, 94 °C and 1 min; annealing, 60 °C and 1 min; extension, 72 °C and 3 min; 25−30 cycles) was performed with the Advantage-GC cDNA PCR kit (Clontech) using 5 ng cDNA as the template and synthetic oligonucleotide primers. The PCR reactions were run on 2% agarose gels and the DNA bands visualized using ethidium bromide.

Expression and Purification of Recombinant Chicken Tau Isoforms. The constructs encoding chicken tau isoforms were prepared by PCR amplification using either cDNA clones or brain cDNA as the template. They were subcloned into the prokaryotic expression vector pRK172 as NdeI/EcoRI fragments, followed by DNA sequencing. Chicken tau was expressed in *E. coli* BL21(DE3), as described (*9*). The bacterial pellets were resuspended in extraction buffer (50 mM Tris-HCl, 0.5 mM EDTA, 0.1 mM DTT, 0.5 mM PMSF, pH 7.4), followed by a 2×1 min sonication (Kontes Micro Ultrasonic Cell Disrupter). The extracts were centrifuged at 27 000*g* for 20 min and the supernatants loaded onto a diethylaminoethyl cellulose (DE52, Whatman) column. The flow-through fraction was then loaded onto a phosphocellullose (P11, Whatman) column equilibrated in extraction buffer. The column was washed in extraction buffer, followed by extraction buffer containing 0.1 M NaCl and 0.2 M NaCl, respectively. Proteins were eluted in extraction buffer containing 0.5 M NaCl, followed by filtration through a 0.45 μm Acrodisc. Following addition of 2-mercaptoethanol to 2%, the protein solution was boiled for 5 min, followed by a 15 min centrifugation at 543 000*g*. The resulting supernatant was dialyzed overnight at 4 °C against a saturated ammonium sulfate solution and centrifuged for 15 min at 100 000*g*. The pellet was resuspended in extraction buffer and dissolved in 6 M guanidine hydrochloride, 50 mM Tris−HCl, pH 7.4, followed by fractionation on an Aquapore RP-300 C8 column (Applied Biosystems) using a gradient of 0.1−0.4% TFA/90% acetonitrile and a flow rate of 0.5 mL/min (*19*).

Microtubule Assembly. Purified recombinant chicken tau (2.5 or 3.5 μM) was incubated with bovine brain tubulin (20 μM, Cytoskeleton) in assembly buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM GTP) at 37 °C. Turbidity was monitored over a 10 min period using a spectrophotometer (Shimazu UV-1601), as described (*19*, *20*). Six separate preparations of each of the eight chicken tau isoforms tested were used.

Preparation of Heat-Stable Chicken Brain Fractions. Brains from chicken embryos (6, 8, 10, 15, and 19 days) and newborn and adult chickens were homogenized in 5 vol of 50 mM Tris−HCl, pH 7.4, 0.5 M NaCl, 0.1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM β-glycerophosphate, 2% 2-mercaptoethanol. Following a 15 min centrifugation at 543 000*g*, the supernatants were boiled for 10 min and recentrifuged, and the supernatants precipitated with 50% ammonium sulfate. The precipitates were then resuspended in 50 mM Tris−HCl, pH 7.4, 0.1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin. Dephosphorylation of chicken tau was carried out using *E. coli* alkaline phosphatase (Sigma Fine Chemicals), as described (*21*). In short, 1 aliq of the heat-stable brain fraction was incubated with 0.8 U/ml alkaline phosphatase in 50 mM Tris−HCl, pH 8.0, 1 mM $MgCl_2$, for 3 h at 65 °C.

Immunoblotting. The polyclonal antibody CTN was raised in a White Dutch rabbit using a synthetic peptide (DITTVE-EQERQHVPSGY) corresponding to residues 7−23 of chicken tau as the immunogen. Antibody BR134 was raised against the carboxy-terminal 14 amino acids of human tau. Antibody Tau1 was purchased from Boehringer Mannheim, whereas anti-phosphopeptide antibodies anti-p202, anti-pT205, anti-pT212, anti-pS214, anti-pS262, anti-pS396, anti-pS404, and anti-pS422 were purchased from Biosource International. The phosphorylation-dependent antibodies AT8, AT100, AT180, and AT270 were purchased from Endogen. Antibody PHF1 was a gift from Dr. P. Davies. Tau1 recognizes tau protein only when the proline-rich region upstream of the repeats is dephosphorylated. The epitopes of AT8, AT100, AT180, AT270, and PHF1 are phospho-S202/T205, phospho-T212/S214, phospho-T231, phospho-T181, and phospho-S396/S404, respectively. The working dilutions of all antibodies were 1:5000−10000. Prior to use, anti-pS202, anti-pS205, anti-pT212, anti-pS214, anti-pS262, anti-pS396, anti-pS404, and anti-pS422 were incubated with recombinant human tau protein (molar ratio 1:200) in 3% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. Immunoblots were developed using the ABC kit (Vectastain) or enhanced chemiluminescence (ECL, Amersham-Pharmacia).

RESULTS

Sequencing of cDNA Clones Encoding Chicken Tau. Six tau clones isolated from a chicken brain cDNA library were sequenced (Figure 1A). Clone 1 was full-length, whereas clones 2−6 encoded only part of the coding region. Clones 1 and 2 comprised a 159 bp insert encoding the 53 amino acid sequence GYPLQIPVDDGSDEPVSETSDAKSTPT-TEDATAPLVEEGDQEDQHGEIPEGTT. This insert is located close to the N-terminus of chicken tau and is 75% identical to the 58 amino acid insert encoded by exons 2 and 3 of the human tau gene (*4*). The characterization of genomic clones for chicken tau showed that the 53 amino acid insert is also encoded by two separate exons, coding

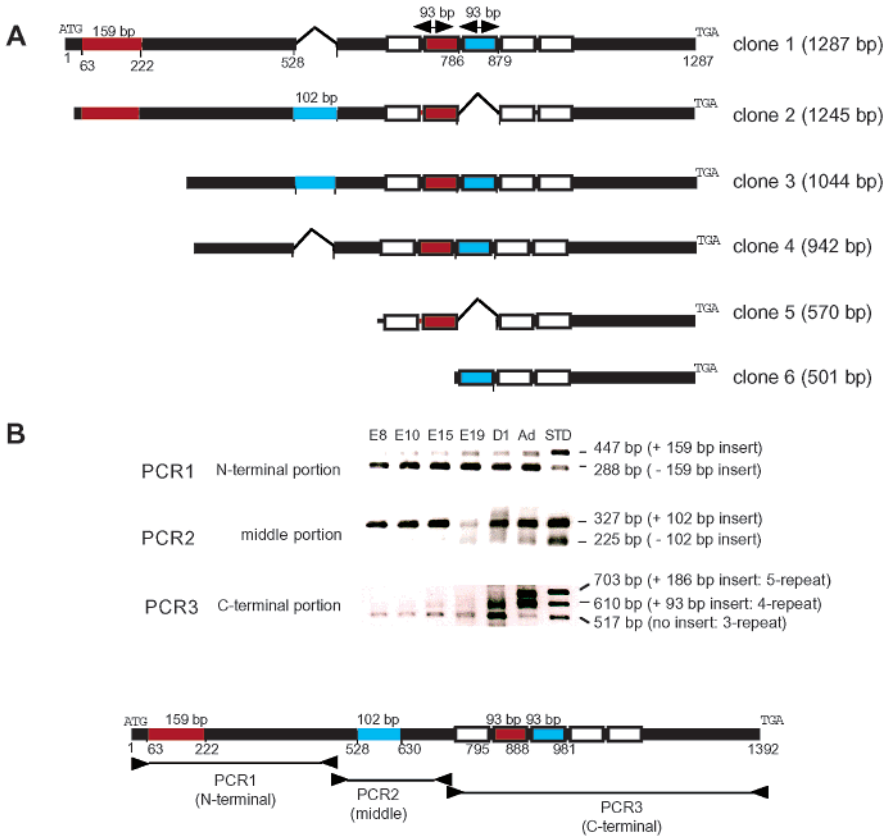


FIGURE 1: (A) Schematic representation of the six chicken brain tau cDNA clones isolated in this study. The alternatively spliced inserts of 159, 102, and 93 bp are shown in red and blue. The inserts shown in red are alternatively spliced in chicken and mammalian brain tau, whereas the inserts shown in blue are only alternatively spliced in chicken brain tau. The tandem repeats are indicated as white, red and blue boxes. The initiation (ATG) and termination (TGA) codons are shown. (B) RT-PCR analysis of the expression of chicken tau as a function of brain development. PCR products were amplified over three regions of chicken tau, the N-terminal (PCR1), the middle (PCR2), and the C-terminal (PCR3) portions. Arrowheads below the schematic diagram of the longest chicken brain tau isoform indicate the positions of the oligonucleotide primers. Total RNA was extracted from brains at embryonic days 8 (E8), 10 (E10), 15 (E15), and 19 (E19), at postnatal day 1 (D1) and at postnatal day 60 (Adult, Ad). The sizes of the different PCR products are indicated on the right.

for 29 and 24 amino acids, respectively (not shown). Clones 2 and 3 differed from the other clones by having a 102 bp sequence upstream of the tandem repeats. It encodes the 34 amino acid sequence GEQPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPP, which is 97% identical to the sequence encoded by part of exon 9 of the human tau gene (*4*). Clones 2 and 5 encode tau proteins with four tandem repeats. Clones 1, 3, 4, and 6 contained a 93 bp sequence that was not present in clones 2 and 5. It is located between the second and the third repeat of the four repeats encoded by clones 2 and 5 and codes for the additional 31 amino acid repeat VQIVNQKLDFSSVQSRCGSKDNIKHIPGGGS. This sequence is 81% identical to that encoded by exon 10 of the human tau gene (*5*). All six cDNA clones encode the 17 amino acid sequence GRKEQKKPPPAAAKPEK, just upstream of the 34 amino acid insert. It is 35% identical to the sequence encoded by exon 8 of the human tau gene (*6*).

RT-PCR was used to determine which inserts are contained in the tau isoforms expressed in chicken brain. To this effect, N-terminal (PCR1), middle (PCR2), and C-terminal (PCR3) portions of chicken tau were amplified using cDNA prepared from brain RNA extracted from various developmental stages (Figure 1B). In the N-terminal portion (PCR1), two PCR products of 288 and 447 bp were obtained. They differed by the 159 bp sequence encoding the 53 amino acid insert located close to the N-terminus of chicken tau. At early developmental stages, all PCR products corresponded to the 288 bp band. As a function of development, the 447 bp band appeared progressively, to become one of the predominant forms in the adult brain. In the middle portion (PCR2), two PCR products of 225 and 327 bp were obtained. They differed by a 102 bp stretch coding for the 34 amino acid sequence homologous to part of exon 9 of the human tau gene. The 327 bp band predominated throughout, with the 225 bp band becoming first detectable at embryonic day 19. In the C-terminal portion (PCR3), three PCR products of 517, 610 and 703 bp were obtained. They code for tau isoforms with either three, four, or five tandem repeats. The 517 bp band was present at all developmental stages and was the only band present up to embryonic day 10. The 610 bp band was first seen at embryonic day 10 and became stronger at later developmental stages. The 703 bp band was first observed after birth and was strong in the adult. Complementary DNA prepared from adult chicken brain showed strong 703 and 610 bp bands and a weak band of 517 bp, consistent with high levels of mRNAs encoding tau isoforms with four and five repeats, and lower levels of mRNA coding for tau isoforms with three repeats.

The cDNA and deduced amino acid sequences of the longest predicted chicken brain tau isoform are shown in Figure 2. It is 463 amino acids in length and contains the alternatively spliced N-terminal 53 amino acid insert, the 34 amino acid insert upstream of the repeats and five tandem repeats. The characterization of genomic clones for chicken

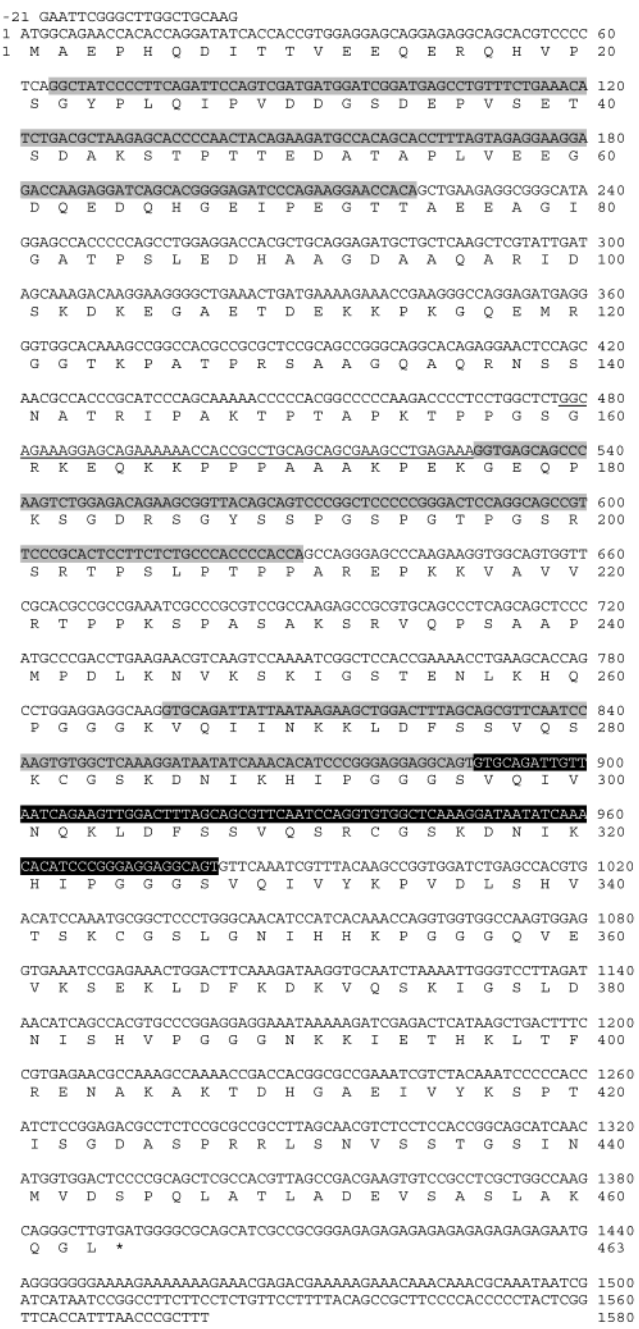


FIGURE 2: Nucleotide sequence of chicken brain tau and deduced amino acid sequence. The predicted amino acid sequence (in single letter code) is shown below the nucleotide sequence of the longest chicken brain tau isoform. Numbers on the right indicate the positions of nucleotides or amino acids. The asterisk denotes the termination codon. Exons which are alternatively spliced are shown in gray and black. The repeat that distinguishes five-repeat tau from four-repeat tau is shown in black.

tau showed that the third repeat is encoded by a separate exon and that the intron separating the exons encoding the second and third repeats is only 145 bp in length (not shown). Overall, the chicken tau amino acid sequence is 74% identical to that of the longest human brain tau isoform (Figure 3). Chicken tau lacks amino acids 22−44 of human tau. This is reminiscent of rodent and bovine tau proteins which lack amino acids 16−25 of human tau (*3*, *8*, *25*). In the carboxy-terminal half, chicken tau is 94% identical to human tau. This region comprises the five tandem repeats of chicken tau. Repeats 1, 2, 4, and 5 of chicken tau can be aligned

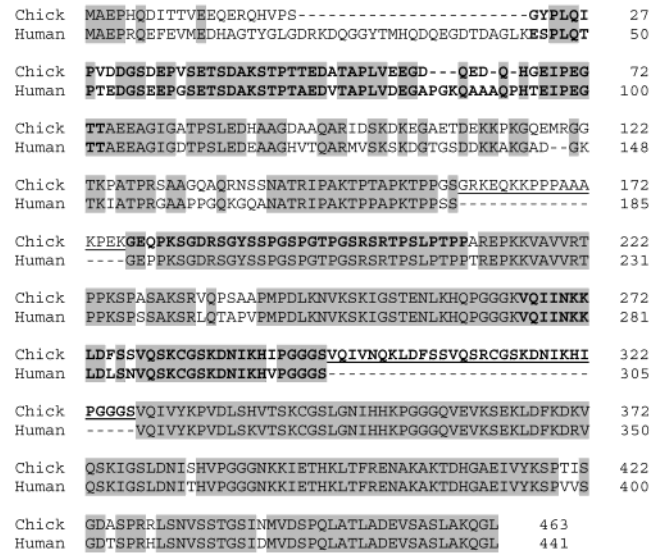


FIGURE 3: Comparison of the amino acid sequences of chicken and human brain tau proteins. The longest brain tau isoforms are shown. Residues that are identical in chicken and human tau are shown in gray. Alternatively spliced sequences are indicated in bold. The underlined sequences are unique to chicken brain tau. Dashes denote gaps introduced into the sequences to maximize the alignment.

with repeats 1−4 of human tau, whereas repeat 3 is unique to the chicken protein. Repeats 1, 2, 4, and 5 of chicken tau are 98% identical in sequence to repeats 1−4 of human tau (Figure 4). The 31 amino acid repeat 3 of chicken tau is most similar to repeat 2, with which it is 90% identical. It is only 48−52% identical with repeats 1, 4, and 5 of chicken tau (Figure 4A). The invertebrate proteins PTL-1 from *C. elegans* and TLP from *D. melanogaster* also carry an extra repeat at an equivalent position to that of the third repeat of chicken tau, with sequence identities of 36−45% between chicken tau and the invertebrate proteins (*14*, *17*, *18*).

Identification of Tau Isoforms in Chicken Brain. On the basis of the RT-PCR results, 12 different tau isoforms could be expressed in chicken brain (Table 1). To determine the true isoform composition in developing and adult brains, we expressed the twelve possible isoforms in *E. coli* and used immunoblotting to compare their gel mobilities with those of dephosphorylated tau from chicken brain (Figure 5, Table 1). Antibody CTN labeled all 12 recombinant tau isoforms. By contrast, antibody Tau1 only labeled the six isoforms containing the 34 amino acid insert. The epitope of Tau1 is contained within this insert (*22*, *23*).

At embryonic day 8, the protein band of 44 kDa apparent molecular mass was predominant. It corresponds to CT3M, a three-repeat tau isoform containing the 34 amino acid insert and lacking the 53 amino acid N-terminal insert. Lower levels of the band with an apparent molecular mass of 59 kDa were also present. It corresponds to CT3NM, a three-repeat tau isoform containing both the 53 and 34 amino acid inserts. At embryonic days 10 and 15, the 44 and 59 kDa bands predominated. In addition, low levels of the band with an apparent molecular mass of 50 kDa were also expressed. It corresponds to CT4M, a four-repeat tau isoform containing the 34 amino acid insert and lacking the 53 amino acid insert. The 59 kDa isoform was the major isoform at embryonic day 19 and postnatal day 1. The 44 and 50 kDa bands were still expressed, albeit at lower levels. In addition, two new

Table 1: Tau Isoforms Expressed in Chicken Brain

isoform	inserts[a] (base-pairs/amino acids) N (159/53)	M (102/34)	R2 (93/31)	R3 (93/31)	length (amino acids)	true MW	apparent MW (kDa)	isoforms detected by immunoblotting[b] E8	E10-E15	E19-D1	adult
CT3	−	−	−	−	314	33025.68	37	−	−	−	−
CT4	−	−	+	−	345	36293.39	42	−	−	−	−
CT5	−	−	+	+	376	39575.05	48	−	−	−	−
CT3M	−	+	−	−	348	36390.20	44	++	++	+	d
CT4M	−	+	+	−	379	39657.91	50	−	+	d	d
CT5M	−	+	+	+	410	42939.57	56	−	−	d	d
CT3N	+	−	−	−	367	38582.18	53	−	−	d	d
CT4N	+	−	+	−	398	41849.89	58	−	−	−	++
CT5N	+	−	+	+	429	45131.5	63	−	−	−	++
CT3NM	+	+	−	−	401	41946.70	59	+	++	++	++
CT4NM	+	+	+	−	432	45214.41	64	−	−	+	++
CT5NM	+	+	+	+	463	48496.07	69	−	−	+	++

[a] N, 53 amino acid N-terminal insert. M, 34 amino acid insert in the middle portion. R2, the second repeat (31 amino acid insert). R3, the third repeat (31 amino acid insert). +, included insert. −, lacked insert. [b] ++, detectable, major isoform. +, detectable. d, detectable on overloaded western blots. −, undetectable.

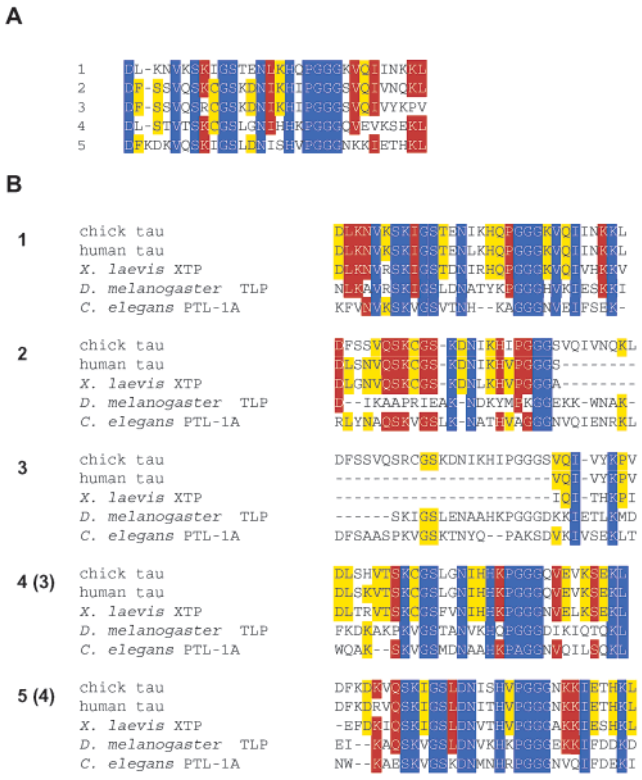


FIGURE 4: Alignment of the five repeats from chicken tau (A), comparison with the four repeats from human tau and *X. laevis* tau-like protein (XTP), and the five repeats from *C. elegans* PTL-1A and *D. melanogaster* tau-like protein (TLP) (B). (A) Amino acids that are identical between three, four, and five repeats are indicated by yellow, red, and blue bars, respectively. (B) Amino acids that are identical between three, four, and five sequences are indicated by yellow, red, and blue bars, respectively. Dashes denote gaps introduced into the sequences to maximize the alignment. The numbers on the left refer to the five repeats of chicken tau, TLP and PTL-1A. Human tau and XTP have only four repeats (bracketed numbers).

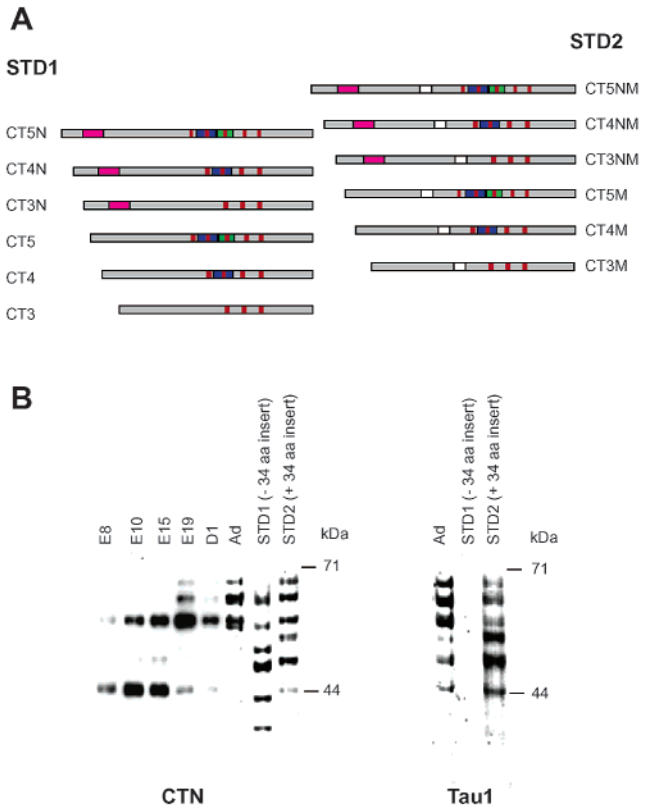
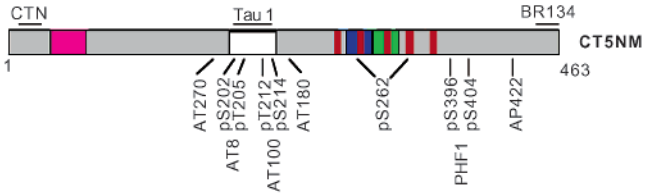


FIGURE 5: Identification of the tau isoforms expressed during chicken brain development. (A) Schematic representation of the 12 tau isoforms predicted from the RT-PCR analysis. STD1 consists of six isoforms that lack the 34 amino acid insert. STD2 consists of six isoforms with the 34 amino acid insert. The alternatively spliced inserts are shown in purple (53 amino acid N-terminal insert), white (34 amino acid insert), blue (second repeat), and green (third repeat). Red boxes denote the tandem repeats (see also Table 1). (B) Immunoblot analysis of dephosphorylated tau from chicken brains taken at embryonic days 8 (E8), 10 (E10), 15 (E15), and 19 (E19), at postnatal day 1 (D1) and at postnatal day 60 (Ad). The recombinantly expressed isoform sets STD1 (six isoforms) and STD2 (six isoforms) were run in parallel. Immunoblotting used either antibody CTN or antibody Tau1.

protein bands were present, with apparent molecular masses of 64 and 69 kDa. They correspond to CT4NM, a four-repeat tau isoform with both the 53 and 34 amino acid inserts, and CT5NM, a five-repeat tau isoform with both the 53 and 34 amino acid inserts.

Finally, in adult chicken brain, five major tau isoforms were recognized by antibody CTN. They had apparent molecular masses of 58, 59, 63, 64, and 69 kDa. By contrast, Tau1 only labeled the 59, 64, and 69 kDa bands, implying that the 58 and 63 kDa bands lack the 34 amino acid insert (not shown). Both isoforms contain the 53 amino acid



antibody	epitope		
	chicken tau	human tau	
CTN	7-23	-	pi
AT270	T155	T181	pd
Tau 1	the proline-rich region upstream of the repeats		pd
pS202	S193	S202	pd
pT205	T196	T205	pd
AT8	S193+T196	S202+T205	pd
pT212	T203	T212	pd
pS214	S205	S214	pd
AT100	T203+S205	T212+S214	pd
AT180	T222	T231	pd
pS262	S253, S378	S262, S356	pd
pS396	S418	S396	pd
pS404	S426	S404	pd
PHF1	S418+S426	S396+S404	pd
AP422	S444	S422	pd
BR134	450-463	428-441	pi

pi: phosphorylation-independent
pd: phosphorylation-dependent

FIGURE 6: Epitope map of anti-tau antibodies. (A) Schematic representation of the longest chicken brain tau isofom (CT5NM), with the positions of epitopes for phosphorylation-dependent and -independent anti-tau antibodies shown. Alternatively spliced inserts are shown in purple (53 amino acid N-terminal insert), white (34 amino acid insert), blue (second repeat), and green (third repeat). Red boxes denote the tandem repeats. (B) Phosphorylated residues and the region in chicken tau and human tau recognized by each anti-tau antibody.

N-terminal insert. The 58 kDa band has four tandem repeats (isoform CT4N), whereas the 63 kDa band has five repeats (isoform CT5N). Thus, of the five tau isoforms expressed in adult chicken brain, one has three repeats (isoform CT3NM), two have four repeats each (isoforms CT4N and CT4NM), and two have five repeats each (isoforms CT5N and CT5NM). They constitute the major adult brain isoforms. On overloaded blots, small amounts of the 44, 50, 53, and 56 kDa bands were also detected, indicating that they constitute minor isoforms in adult brain (not shown).

Developmental Regulation of Tau Phosphorylation in Chicken Brain. Phosphorylation-dependent anti-tau antibodies were used to investigate the phosphorylation of tau from chicken brain as a function of development (Figure 7). Several antibodies strongly labeled tau throughout development but gave only a very weak or no signal with tau from adult chicken brain. This was the case of AT8, anti-pT205, anti-pT212, anti-pS262, and anti-pS422. Antibody Tau1, which recognizes dephosphorylated tau, only labeled tau from newborn and adult chicken brains. By contrast, the phosphorylation-dependent anti-tau antibodies AT270, anti-pS202, AT180, PHF1, anti-pS396, and anti-pS404 gave a strong signal with tau from both developing and adult chicken brains. Two phosphorylation-dependent antibodies, AT100 and anti-pS214, failed to label tau from any of the stages studied (not shown).

Effects of the Alternatively Spliced Inserts on the Ability of Chicken Tau to Promote Microtubule Assembly. To examine the effects of the number of tandem repeats, we compared recombinant tau protein isoforms CT3NM (three repeats), CT4NM (four repeats) and CT5NM (five repeats). Each isoform contains the 53 and 34 amino acid inserts. As shown in Figure 8A, the extent of polymerization with CT5NM was about 1.2 and 1.8 times higher than that with CT4NM and CT3NM, respectively. There were also marked differences between the isoforms when the rate of microtubule assembly was expressed as the optical density at 1 min (5% for CT3NM, 68% for CT4NM, with the value for CT5NM taken as 100%). Similar differences between repeats were obtained with isoforms CT3N (three repeats), CT4N (four repeats), and CT5N (five repeats), which contain the 53 amino acid insert but lack the 34 amino acid insert (Figure 8B). However, the extent of polymerization with isoforms CT3N, CT4N, and CT5N was less than with isoforms CT3NM, CT4NM, and CT5NM. To examine the influence of the 53 and 34 amino acid inserts on the ability of chicken tau to promote microtubule assembly, we compared the three-repeat-containing isoforms CT3M (34 amino acid insert), CT3N (53 amino acid insert), and CT3NM (53 and 34 amino acid inserts). As shown in Figure 8C, the extent of polymerization with CT3NM was approximately 1.4 and 1.2 times higher than that with CT3N and CT3M, respectively. When the results were expressed as the optical density at 1 min, there was no difference between isoforms CT3M and CT3NM. By contrast, isoform CT3N showed a much slower rate of microtubule assembly (13%, with the values for CT3M and CT3NM taken as 100%).

DISCUSSION

Tau proteins from several mammalian species have been characterized at the molecular level (*3*−*5*, *8*, *24*−*28*). Here we present the first report on the structure and function of tau from a nonmammalian vertebrate. The longest isoform of tau from chicken brain comprises 463 amino acids (isoform CT5NM). Like its mammalian counterparts, it contains several alternatively spliced inserts. The most N-terminal insert is 53 amino acids long and is present in a position equivalent to that of the 58 amino acid insert encoded by exons 2 and 3 in human tau (*4*), with which it is 75% identical. As in mammalian tau, the chicken insert is encoded by two separate exons that code for 29 and 24 amino acids, respectively. However, unlike mammalian tau, the 29 amino acid insert is not expressed in the absence of the 24 amino acid insert. In chicken tau, a 34 amino acid sequence that is located upstream of the repeat region is also alternatively spliced. It is 97% identical to the beginning of the sequence encoded by exon 9 of the human tau gene (residues 186−219). This sequence is rich in phosphorylation sites and contains five S/T−P sites. It contains the epitopes of a number of phosphorylation-dependent anti-tau antibodies, such as Tau1, pS199, AT8, pT212, pS214, and AT100. Immediately upstream of the 34 amino acid insert, tau isoforms from chicken brain contain a sequence of 17 amino acids that is rich in lysine, proline, and alanine residues. This sequence, which is encoded by exon 8 of the human tau gene (*6*), is not expressed in rodent or human brain (*29*). It has been described in some cDNA clones encoding rhesus monkey and bovine tau proteins (*25*, *26*, *28*). However, the

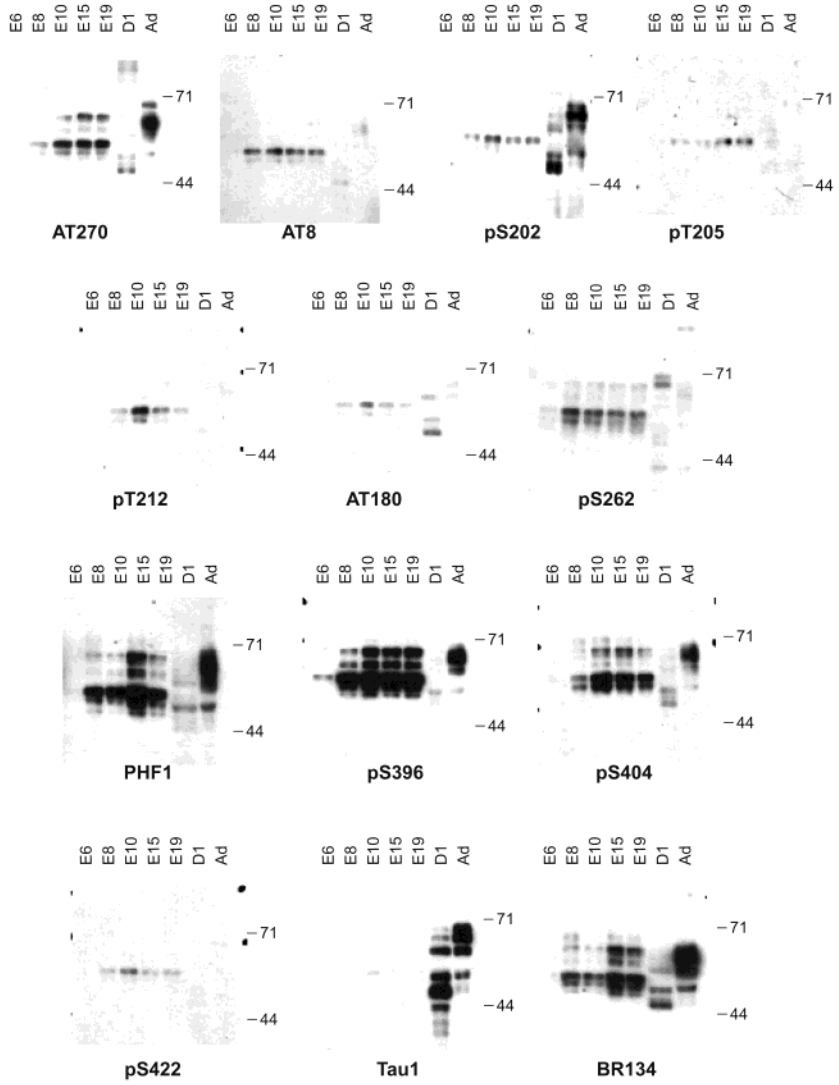


FIGURE 7: Phosphorylation of tau protein during chicken brain development. Equal aliquots of heat-stable fractions prepared from chicken brains taken at embryonic days 6 (E6), 8 (E8), 10 (E10), 15 (E15), and 19 (E19), at postnatal day 1 (D1) and at postnatal day 60 (Ad) were subjected to immunoblotting using 12 phosphorylation-dependent anti-tau antibodies (AT270, AT8, pS202, pT205, pT212, AT180, pS262, PHF1, pS396, pS404, pS422, and Tau1) and the phosphorylation-independent anti-tau antibody BR134. The numbers on the right indicate the apparent molecular mass (in kDa).

major tau isoforms from bovine brain lack the sequence encoding exon 8 (*25*, *26*, *29*). The constitutive expression of the 17 amino acid sequence, in conjunction with the variable expression of the 34 amino acid insert that follows it, represents a major difference between mammalian and chicken tau proteins.

Like mammalian tau sequences, some chicken tau isoforms contain three or four tandem repeats. In all species, the additional alternatively spliced repeat is located after the first repeat. Unlike mammalian tau, isoforms with five repeats are also expressed in the chicken brain. The alternatively spliced extra repeat follows the second repeat with which it is 90% identical. It is only 48−52% identical with repeats 1, 4, and 5, suggesting that it may have arisen through duplication of the exon that encodes the second repeat. Previously, five repeats have been described in the tau-like proteins from invertebrates (*14*, *17*, *18*). The additional repeat in *C. elegans* and *D. melanogaster* is located in a position equivalent to that of the third repeat in chicken tau, with which it is 36−45% identical. In human tau, 18 amino acid positions are known at which an amino acid change or the deletion of a single amino acid causes FTDP-17. Seventeen of these amino acids are conserved in chicken tau. The only exception is the amino acid at position 5, which is an arginine in human tau and a histidine in chicken tau. A late-onset form of FTDP-17 has been shown to be associated with a R5H mutation in the human tau gene (*30*). A second mutation at this codon, R5L, has been described in a case of progressive supranuclear palsy (*31*).

The expression of tau isoforms in chicken brain is subject to developmental regulation. By RT-PCR, transcripts encoding three-repeat tau predominate during development inside the egg, with transcripts encoding four-repeat tau first appearing during the later stages of egg development. Transcripts encoding five-repeat tau appear only after hatching. Both four- and five-repeat transcripts constitute the predominant species in adult chicken brain. Transcripts encoding the 53 amino acid insert appear as a minor form during egg development, but come to predominate in the adult brain. Throughout development, most tau transcripts encode the 34 amino acid insert, with transcripts lacking this insert appearing during the later stages of egg development.

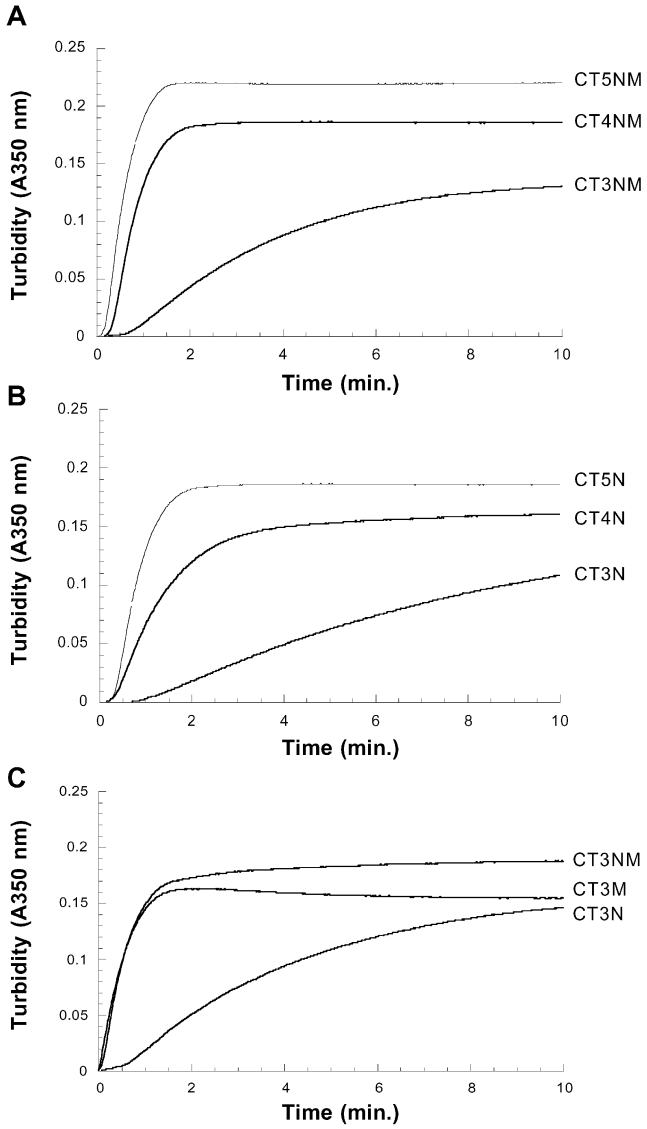


FIGURE 8: Tubulin polymerization in the presence of chicken brain tau isoforms. (A) Polymerization of tubulin induced by three-repeat (CT3NM), four-repeat (CT4NM), and five-repeat (CT5NM) chicken tau. These isoforms contain both the 53 and the 34 amino acid inserts. (B) Polymerization of tubulin induced by three-repeat (CT3N), four-repeat (CT4N), and five-repeat (CT5N) chicken tau. These isoforms contain the 53 amino acid insert but lack the 34 amino acid insert. (C) Polymerization of tubulin induced by three-repeat chicken tau isoforms with the 53 amino acid insert but without the 34 amino acid insert (CT3N), with the 34 amino acid insert but without the 53 amino acid insert (CT3M), and with both the 53 amino acid and 34 amino acid inserts (CT3NM). Tubulin (20 μM) was incubated with tau at 2.5 μM (A and B) or 3.5 μM (C). Tubulin polymerization was monitored by an increase in turbidity over time. A typical experiment is shown for each panel. Similar results were obtained in six separate experiments.

These findings at the RNA level are reflected at the protein level. At embryonic day 8, three-repeat tau with the 34 amino acid insert predominates (isoform CT3M). At later stages, isoforms with four repeats and the 53 amino acid insert appear. Four-repeat tau with the 53 and 34 amino acid inserts is the major isoform (CT4NM) at the time of hatching. In adult chicken brain, five isoforms account for the bulk of tau. One isoform (CT3NM) has three repeats, two isoforms (CT4N and CT4NM) have four repeats each, and two isoforms (CT5N and CT5NM) have five repeats each. These isoforms contain the 53 amino acid insert, but two of them (CT4N and CT5N) lack the 34 amino acid insert.

Broadly speaking, the developmental regulation of tau isoforms in the chicken brain resembles that in mammals, in that three-repeat tau without an N-terminal insert predominates at early developmental stages, with four-repeat tau containing the N-terminal insert being expressed later in development (*5*, *27*). However, in marked contrast to mammals, five-repeat tau is a major species in the adult chicken brain. Overall, the developmental changes in the expression of tau isoforms occur earlier in the chicken brain than in rodent and human brains.

The phosphorylation of tau is developmentally regulated in chicken brain, similar to what is seen in mammalian brain. Chicken tau is phosphorylated more extensively during development in the egg than after hatching. It is least phosphorylated in the adult brain. Several sites, such as T205, T212, and S422, are phosphorylated early in development, but not in the adult. The same is true of rat brain (*32*, *33*). Other sites, such as T181, S202, T231, S396, and S404, are phosphorylated in developing and adult brains, in confirmation of previous results in chicken brain (*34*−*36*). Similar findings have also been reported in mammalian brain (*37*). S214 in tau is not phosphorylated in either chicken or mammalian brain. It is, however, phosphorylated in the filamentous tau found in human neurodegenerative diseases and transgenic mouse models thereof (*38*−*40*). Thus, phosphorylation at this site appears to be diagnostic of filamentous tau. Phosphorylation negatively regulates the ability of tau to interact with microtubules (*41*). The developmentally regulated phosphorylation of tau that is observed in chicken and mammalian brains is believed to be important for ensuring a flexible cytoskeleton during development.

The number of tandem repeats positively influences the ability of *C. elegans* and human tau proteins to promote microtubule assembly (*9*, *14*). The same is true of chicken tau, where five-repeat tau had the greatest ability to promote microtubule assembly, followed by tau proteins with four and three repeats. In chicken tau, the presence of the 34 amino acid insert significantly increased the rate of microtubule assembly and the extent of polymerization. This is in line with findings on human tau, suggesting that the proline-rich region upstream of the repeats is of functional significance (*42*, *43*). By contrast, the 53 amino acid insert did not influence the rate of microtubule assembly, similar to what has been described for the 58 amino acid N-terminal insert in human tau (*9*). However, its presence increased the extent of microtubule assembly. The assay used was an unseeded assembly assay. Therefore, the measured increase in turbidity represented a combination of microtubule nucleation and microtubule elongation reactions.

Previously, tau-like proteins were molecularly cloned from *C.elegans*, *D. melanogaster* and *X. laevis* (*4*, *17*, *18*, *44*). They contain carboxy-terminal repeats similar to those in tau but show otherwise only few sequence similarities with chicken and mammalian tau proteins. Mammalian genomes contain three genes encoding proteins of the tau family (*tau*, *MAP2* and *MAP4*), with *C. elegans* and *D. melanogaster* having only one gene encoding a protein with tau-like repeats. Thus, radiation of the tau family may have occurred after the vertebrate and invertebrate lines separated during evolution. It is interesting to note that the human tau gene

on chromosome 17 is present within a segment that is duplicated in the region of chromosome 2 that contains the MAP2 gene (*45*). The fact that the tandem repeat sequences are well conserved over a long period of evolution shows that they are of great functional importance. It remains to be determined whether the presence of isoforms with five tandem repeats is a general characteristic of tau protein in birds. It will be interesting to characterize tau proteins from fishes at the molecular level.

The isoform composition of tau proteins in adult brain is not conserved between species. Thus, chicken tau has isoforms with three, four, and five tandem repeats, rodent tau only consists of isoforms with four repeats (*15*, *27*), whereas human tau consists of isoforms with three and four repeats (*4*). The number of tandem repeats is thus unlikely to be of crucial importance for the normal function of tau in the adult brain. This notwithstanding, at least in human brain, a correct ratio of tau isoforms is of crucial importance, since the relative overproduction of four-repeat tau that is caused by some mutations in the tau gene leads to tau filament formation, neurodegeneration and dementia in mid-life (*2*, *13*).

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