A Novel Tau Transcript in Cultured Human Neuroblastoma Cells Expressing Nuclear Tau

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Abstract. We previously reported the presence of the microtubule-associated protein, tau in the nuclei of primate cells in culture. The present study confirms the existence of nuclear tau in two human neuroblastoma cells lines by indirect immunofluorescence and Western blot using mAbs to tau. Northern blot analysis of poly A$^+$ mRNA detects a novel 2-kb tau transcript coexpressed with the 6-kb message in cultured human cells and human frontal cortex. PCR and cDNA sequencing demonstrate that the 2-kb message contains the entire tau coding region. Furthermore, actinomycin D transcription inhibition experiments indicate that the 2-kb message is not derived from the 6-kb message, but instead arises from the original tau transcript. One of the human neuroblastoma cell lines examined contains both nuclear and cytoplasmic tau as assayed by both Western blot and indirect immunofluorescence. Northern blot analysis of this cell line indicates that copious amounts of the 2-kb message are present while little of the 6-kb transcript is obvious. Immunofluorescence analysis of this cell line demonstrates that the cytoplasmic tau is not localized to microtubules. Together, these results indicate that the 2-kb tau message in humans may specify tau for non-microtubule functions in both the cytoplasm and the nucleus. We hypothesize that this is accomplished via a message targeting mechanism mediated by the untranslated regions of the tau messages.

TAU polypeptides are microtubule-associated proteins (MAPs)[1] found in abundance in the nervous system of all vertebrates (Weingarten et al., 1975; Connolly et al., 1977; Cleveland et al., 1977; Binder et al., 1985). Most published observations indicate that tau is largely an axonal protein (Binder et al., 1985; Peng et al., 1986; Kowall and Kosik, 1987; Migheli et al., 1988; Brion et al., 1988) although others have demonstrated its presence within the somatodendritic compartment of neurons (Papasozomenos and Binder, 1987; Migheli et al., 1988; Riederer and Innocenti, 1991). In support of these observations, in situ hybridization studies using labeled antisense tau cDNA detected tau mRNA not only within the somata but also within the proximal dendrites of large cortical neurons (Kosik et al., 1989a), suggesting the occurrence of local tau synthesis (reviewed in Steward and Banker, 1992). Moreover, immunohistochemical studies have further demonstrated the presence of tau within perineuronal glia, astrocytes, and interfascicular oligodendrocytes (Couchie et al., 1985; Papasozomenos and Binder, 1987; Migheli et al., 1988; Riederer and Innocenti, 1991). Taken together, these reports indicate that tau is found in most cell types within the central nervous system of mammals.

The function of tau is generally thought to involve microtubule stabilization. Early studies established tau as a stimulatory agent for in vitro microtubule assembly (Weingarten et al., 1975). Subsequently, Drubin and Kirschner (1986) microinjected purified tau into fibroblasts and documented increased stability of the endogenous microtubules to disassembly by antimicrotubule drugs. Ultrastructural localization studies in rat brain suggested that tau was perhaps more than just a MAP. As expected, tau was present on both axonal and somatodendritic microtubules in situ; however, the non-microtubule localization of tau to ribosomes was detected within the somata of neurons suggesting another functional role for this protein (Papasozomenos and Binder, 1987).

The biochemical nature of tau is complex, due in part to its multiple isoform composition. The electrophoretic pattern characteristic of tau proteins in the central nervous system is heterogeneous in nature largely as a result of alternate splicing of a single mRNA transcript resulting in a 6-kb message that is translated into 5-7 tau isoforms of 45-65 kD (Lee et al., 1988; Himmler et al., 1989; Himmler, 1989). Each

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1. *Abbreviations used in this paper:* AD, Alzheimer's Disease; MAP, microtubule-associated protein; NOR, nucleolar organizer regions; PNS, peripheral nervous system; UTR, untranslated regions.

tau isoform contains either three or four stretches of 31- or 32-amino acid repeats in the carboxy-terminal half of the molecule representing the microtubule binding domains (Aizawa et al., 1988; Lee et al., 1989). Amino-terminal insertions of either 29 or 58 amino acids combine with either three or four repeats resulting in the six human tau isoforms (Goedert et al., 1989). In their nonphosphorylated state, these isoforms can be readily separated by electrophoresis; however, phosphorylation can alter electrophoretic mobilities and complicate this pattern considerably (Lindwall and Cole, 1984a,b; Butler and Shelanski, 1986; Baudier and Cole, 1987; Kanai et al., 1989; Steiner et al., 1990). Additionally, an 8-kb message has been described in the peripheral nervous system (PNS) that codes for proteins of 110–130 kD (Oblinger et al., 1991). cDNAs encoding this PNS tau have been cloned and sequenced in rats (Goedert et al., 1992b) and in mice (Couchie et al., 1992). The derived sequences from the rat and mouse cDNAs contained inserts of 254 and 237 amino acids, respectively. Additionally the mouse PNS tau sequence also contained an additional 66 residues with homology to bovine exon 6 (Couchie et al., 1992; Himmler et al., 1989; Himmler, 1989). These inserts explain the increase in apparent molecular weight displayed by PNS tau.

Interest in tau proteins has increased recently due to its involvement in Alzheimer's Disease (AD) pathology. The abnormal paired helical filaments (PHFs) which comprise the neurofibrillary tangles that accumulate in specific neuronal populations in brains of AD patients appear to be polymers of tau (Lee et al., 1991; Greenberg et al., 1992). Additionally, tau within the PHFs has been shown to be abnormally phosphorylated (Grunke-Iqbal et al., 1986a; Ksiezak-Reding et al., 1990; Greenberg et al., 1992), although splicing events within the coding region of the tau mRNA seem identical in both PHF and normal tau (Goedert et al., 1992a). Abnormally phosphorylated tau has also been found to localize on ribosomes in neurons and astrocytes in AD (Papasozomenos, 1989). These findings implicate tau in the cascade of events leading to neuronal death in the vulnerable brain regions during the course of AD. Neuronal death in the hippocampus, frontal cortex, and superior temporal gyrus is presumed to lead to the memory and cognitive deficits characteristic of human senile dementia.

Previously, we demonstrated the existence of nuclear tau isoforms in cultured primate cells (Loomis et al., 1990). Nuclear tau was associated with both the fibrillar regions of interphase nucleoli and the nucleolar organizer regions (NORs) of mitotic chromosomes. These areas represent the location of the rRNA genes during transcription and cell division, respectively. As well, nuclear tau appeared extremely insoluble, requiring the use of formic acid and SDS for solubilization, much like the PHF tau described above. Although only the Tau-1 mAb detected nuclear tau isoforms by indirect immunofluorescence, other tau mAbs, whose epitopes span the molecule (Kosik et al., 1988), recognized many characteristic tau polypeptides on immunoblots of solubilized nuclei, indicating the presence of multiple tau isoforms.

The results described in this report extend our previous findings concerning the nature of nuclear tau. The presence of nuclear tau is documented in two human neuroblastoma cell lines by indirect immunofluorescence and Western blot analysis. Additionally, a novel 2-kb tau transcript coex-

pressed with the 6-kb message is described in human cells in culture and brain tissue isolated from frontal cortex. The 2-kb message contains the entire tau coding region and actinomycin D transcription inhibition experiments indicate that it derives, not from further processing of the 6-kb message, but from the original tau transcript. Northern blot analysis of a human cell line which contains both nuclear and cytoplasmic tau demonstrates that the majority of the tau message is the 2-kb species. Interestingly little, if any, of the cytoplasmic tau in this cell line localizes to microtubules. Together, these results indicate that the 2-kb tau message in humans may specify tau for nonmicrotubule functions in both the cytoplasm and the nucleus.

Materials and Methods

Cell Culture

The cell lines N2A (mouse neuroblastoma) and JC (human neuroblastoma) were cultured in DME supplemented with 10% FBS (Gemini). CG cells (human neuroblastoma) were cultured in DME supplemented with 5% horse serum (GIBCO BRL, Gaithersburg, MD). CHO cells were cultured in McCoy's 5A medium supplemented with 10% CPSR-4 (Controlled Process Serum Replacement; Sigma, St. Louis, MO). All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Mitotic Cell Preparations

Cells were incubated in 0.1 μg/ml of Colcemid for 12 h. Mitotic cells were dislodged from the culture flask by gently shaking the flask. Subsequently, the cells were pelleted by centrifugation (1,000 g for 5 min), resuspended in PBS, pH 7.4, and washed twice in PBS. The mitotic cells were swollen in hypotonic (0.075 M KCl) for 5 min at 37°C and then cytospun onto glass coverslips at 1,500 rpm for 2 min using a Shandon Cytospin (Shandon Southern Instruments Inc., Sewickley, PA), and then fixed in 3% EM grade formaldehyde (Tousimis Res. Co., Rockville, MD) in PBS for 30 min at 24°C.

Indirect Immunofluorescence

Cells cultured on glass coverslips and fixed with 3% formaldehyde for 30 min at 24°C and mitotic cell preparations described above were processed for indirect immunofluorescence microscopy as previously described (Loomis et al., 1990). Briefly, the cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min, rinsed twice in PBS, reduced in 0.5 μg/ml NaBH₄ in PBS for 4 min, and blocked with 1% BSA in PBS for 10 min. To determine if JC cells contained microtubule-associated tau, the cells were detergent extracted in microtubule-stabilizing buffer (to remove soluble cytoplasmic proteins) prior to formaldehyde fixation using the protocol of Steuer et al. (1990). Briefly, cells on coverslips were rinsed in PBS, placed in PEEM (0.1 M Pipes, pH 6.9, 1 mM MgSO₄, 0.1 mM EDTA, and 2 mM EGTA) to stabilize microtubules and then placed in 0.5% Triton X-100 in PEEM containing 0.1 mM PMSF and 1 mg/ml each of chymostatin, leupeptin, antipain, and pepstatin. The cells were then stained with the Tau-1 IgG mAb (1:40) followed by fluorescein-conjugated IgG specific sheep antimouse and rabbit anti-sheep antibodies respectively (1:20; Boehringer Mannheim Biochemicals, Indianapolis, IN). For tau–microtubule double-label preparations, cells were further processed using a monoclonal IgM antibody to tubulin (5H1) followed by a goat anti-mouse IgM specific secondary antibody conjugated to Texas red. All antibody incubations were for 30 min at 37°C in a humidified atmosphere. Each incubation was followed by three 5-min washes in PBS. The preparations were counterstained with 1 μg/ml Hoechst 33258 (Sigma) in PBS for 5 min to visualize the DNA. The processed coverslips were mounted onto glass slides with a 10:1 (vol/vol) mixture of glycerol/PBS containing 0.1% p-phenylenediamine (Sigma) to reduce photobleaching, and then sealed with nail polish.

Computer-enhanced Video Microscopy and Image Analysis

Cells processed for indirect immunofluorescence were analyzed using a Nikon Optiphot epifluorescence microscope equipped with differential in-

terference contrast (DIC) optics and a Dage series 68 SIT video camera (Dage-MTI Inc., Wabash, WI) coupled to an Image-1 image analysis system (Universal Imaging, Westchester, PA). Image processing was performed by setting the gain and black level on both the computer and camera to provide an optimal signal. Then 128 video frames were summed into the foreground frame buffer, the image was defocused, and 128 video frames were summed into the background frame buffer which was subsequently subtracted from the foreground buffer. The resultant image was subjected to further contrast enhancement. Overlays of double fluorescence were obtained by aligning processed images in separate frame buffers using discernible fiduciary points within the images and then superimposing the images by adding them together using the "average" logic function of Image-1. The images were printed with a Sony UP-5000 color video printer (Sony, Montvale, NJ).

Nuclear Isolation and Extraction

Nuclear and cytoplasmic fractions from the various cell lines were isolated as described by Mitchison and Kirschner (1985). The isolated nuclei were washed, resuspended in 50 mM Tris, pH 6.8, sonicated for 30 s, extracted with 2.5% formic acid for 60 s and then centrifuged in a microfuge for 2 min to pellet insoluble material. The supernatant was then desalted into 25 mM Tris-HCl (pH 6.8) and solubilized by boiling in electrophoresis sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol; Laemmli, 1970). Cytoplasmic fractions were concentrated using an ultrafiltration device (Diaflo, YM10 filter; Amicon Corp., Arlington Heights, IL) prior to solubilization with electrophoresis sample buffer as described above. The purity of nuclear and cytoplasmic fractions was determined by phase microscopy and fluorescence microscopy using the DNA binding dye Hoechst 33258. Additionally, the purity of the nuclear fractions was confirmed by immunoblotting with mAbs to tubulin as previously described (Loomis et al., 1990).

SDS-PAGE and Western Blotting

Protein concentrations were determined by a modification of the method of Lowry et al. (1951). 25 μg of an extract from human frontal cortex, 75 μg of nuclear extracts from all the cell lines, 50 μg of the cytoplasmic extracts from CHO, CG, and JC and 25 μg of the cytoplasmic extract from N2A were separated by SDS-PAGE using a 5–12.5% linear polyacrylamide gradient gel (Laemmli, 1970). Less total N2A cytoplasmic protein was loaded because N2A cells expressed more tau than the other cell lines assayed. After electrophoresis, proteins were transferred to nitrocellulose (Towbin et al., 1979) and probed with the mouse mAb, Tau 46.1. Bound antibodies were detected using a peroxidase-conjugated secondary antibody followed by reaction with H_2O_2 and an appropriate chromogen as described previously (Binder et al., 1985).

mRNA Isolation and Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Poly A^+ mRNA was isolated from cultured cells by the method of Badley et al. (1988) and separated by electrophoresis on denaturing 1% low melt agarose gels containing 2.2 M formaldehyde (Sambrook et al., 1989). The 2- and 6-kb regions of the resultant RNA gels were excised, the mRNA extracted with phenol/chloroform, precipitated with ethanol, and reverse transcribed to cDNA using the 1st Strand Synthesis Kit from Stratagene (La Jolla, CA). PCR was performed with Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT) using the conditions specified by the manufacturer. Briefly, 5 μl of cDNA from the RT-cDNA pool was amplified by adding 25 pmol of each primer, 200 μM of each dNTP, 1× reaction buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1% gelatin), and 2.5 U of Taq polymerase in a final volume of 100 μl. The samples were subjected to 30 cycles of denaturation (1 min at 94°C), annealing (2 min at 55°C) and extension (3 min at 72°C) using a DNA Thermal Cycler 480 (Perkin Elmer/Cetus). The products of the reaction were analyzed on a 1% agarose/ethidium bromide gel. PCR was first performed with two primers (nucleotides [-6]-12 and nucleotides 1308-1326) to amplify the whole coding region of human tau. The nucleotide numbers listed here and below refer to the longest human tau isoform (441 amino acids) containing the fourth repeat in the microtubule binding domain (exon 10) as well as exons 2 and 3 in the amino end of the tau molecule (Goedert et al., 1989). The resultant products could not be seen with ethidium bromide stain but were detected by Southern blots (data not shown). These low abundance cDNAs were reamplified with two primer pairs that amplify from the beginning of exon 1 (nucleotides [-6]-12)

to the middle of exon 9 (nucleotides 669-696) as well as from the middle of exon 9 (nucleotides 669-696) to the 3' end of the tau coding region (nucleotides 1308-1326). The primers used within exon 9 provided an overlap region to assure that the entire coding region of tau was present in the 2-kb message. The PCR fragments generated from the 2-kb mRNA were separated on a 1% agarose gel, stained with ethidium bromide, and the appropriate band was excised, purified and ligated into the sequencing vector (TA cloning™ from Invitrogen). DNA sequencing was performed by the dideoxy-chain termination method using the Sequenase DNA sequencing kit (version 1.0; United States Biochemical Corp., Cleveland, OH).

Northern Blot Analysis

Equal amounts of poly A^+ mRNA extracted and purified as outlined above were loaded onto denaturing RNA gels and, after electrophoresis, transferred to an Immobilon membrane (Millipore Corp., Milford, MA). Hybridization was performed for 20 h at 42°C in 50% formamide, 5× SSC, 5× Denhardt's, 1% SDS, and 100 μg/ml salmon sperm DNA using a human three repeat tau cDNA (courtesy of Dr. Gloria Lee, Harvard Medical School, Cambridge, MA) labeled by nick-translation with α-^{32}P-dCTP. After washing, the blots were dried and then autoradiographed at -70°C for 1-2 d.

Transient Transfection of Tau cDNA

CHO cells which have been shown not to express tau were plated on coverslips. The pEnl23c eukaryotic expression vector (generously provided by Dr. Gloria Lee) containing the whole coding region of three repeat short human tau (lacking exons 2, 3, and 10) was transfected into cultured CHO cells using the LIPOFECTIN reagent (GIBCO BRL). The CHO cells were cultured in a six-well plate until 40–60% confluent. 4 μgs of DNA was added with the LIPOFECTIN reagent according to the manufacturer's protocol. The cells were incubated for 6 h at 37°C and then the DNA-LIPOFECTIN–containing medium was removed. The cells were cultured in fresh serum-containing medium for an additional 42 h before fixation and processing for IF using the Tau-1 mAb.

Results

Previous work from our laboratory demonstrated the presence of nuclear tau in two human neuroblastoma cell lines. Immunofluorescence analysis of several cell lines representing various species indicated that nuclear tau existed only in primates (Loomis et al., 1990). We undertook the current study to confirm the presence of nuclear tau at the molecular level in two human neuroblastoma cell lines (CG and JC) and to further test for this form of tau in cell lines of mouse neuroblastoma (N2A) and CHO.

Indirect Immunofluorescence Analysis of Tau in Cultured Cells

Nuclear tau was observed by indirect immunofluorescence (IF) using the Tau-1 mAb. This antibody stained the fibrillar regions of interphase nucleoli in both human neuroblastoma cell lines tested (Fig. 1, A, A', B and B', arrowheads). One of these cell lines (JC) exhibited both cytoplasmic and nucleolar staining (Fig. 1, B and B'), while in the other (CG), fluorescence was observed only in the nucleolus (Fig. 1, A and A', arrowheads). The mouse neuroblastoma cell line (N2A) revealed intense cytoplasmic Tau-1 IF while its nucleolus remained unlabeled (Fig. 1 C, and C'). A convenient negative control was provided by CHO cells which showed no Tau-1 immunostaining in either the nucleolus or the cytoplasm (Fig. 1, D and D').

Since Tau-1 immunofluorescence was observed in the fibrillar regions of interphase nucleoli in human cell lines, chromosome spreads from each of these lines were stained with the Tau-1 mAb to determine the location of nuclear tau
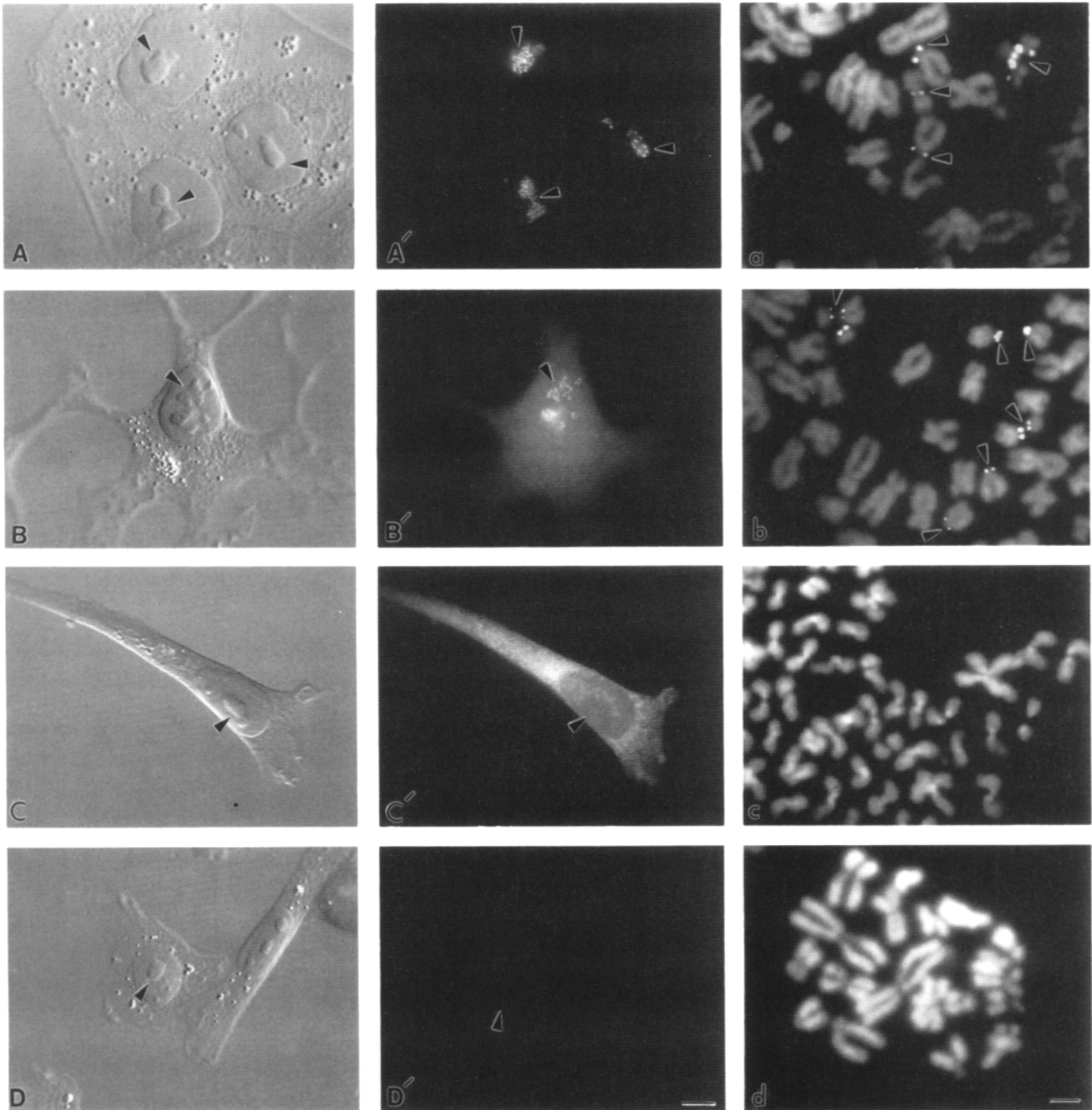
Figure 1. Localization of tau by indirect immunofluorescence in interphase cells and mitotic chromosomes of CG (*A*, *A'*, and *a*) and JC (*B*, *B'*, and *b*) human neuroblastoma, N₂A murine neuroblastoma (*C*, *C'*, and *c*) and CHO (*D*, *D'*, and *d*) cell lines. Arrowheads in the immunofluorescence images (*A'*, *B'*, *C'*, and *D'*) correspond to the arrowheads that denote the nucleolus in the DIC images (*A*, *B*, *C*, and *D*). Both CG (*A* and *A'*) and JC (*B* and *B'*) cell lines displayed tau staining in the dense fibrillar regions of the nucleolus as well as staining of the NORs present on the acrocentric chromosomes (*arrowheads*, *a* and *b*). Immunolocalization of tau in the cytoplasm was detected in JC neuroblastoma (*B'*) and N₂A cells (*C'*). Tau immunoreactivity was not detected in the cytoplasm or nucleus of CHO cells (*D'*). As expected, nuclear tau was not detected on the chromosomes from N₂A and CHO cell lines (*c* and *d*). Bar: (*A* and *A'–D* and *D'*) 30 μm; (*a–d*) 15 μm.

during mitosis. As expected, the JC and CG human neuroblastoma cells, which exhibited interphase nucleolar staining (Fig. 1, *A'* and *B'*), also contained Tau-1 reactivity on the NORs present on the short arms of the acrocentric chromosomes (# 13, 14, 15, 21, and 22; see Fig. 1, *a* and *b*, *arrow-*

heads). In contrast, chromosome spreads from both N₂A and CHO cells, which exhibited no interphase nucleolar staining (Fig. 1, *C'* and *D'*), failed to react with the Tau-1 antibody by IF (Fig. 1, *c* and *d*). As previously reported, Tau-1 was the only mAb that displayed nucleolar and NOR im-
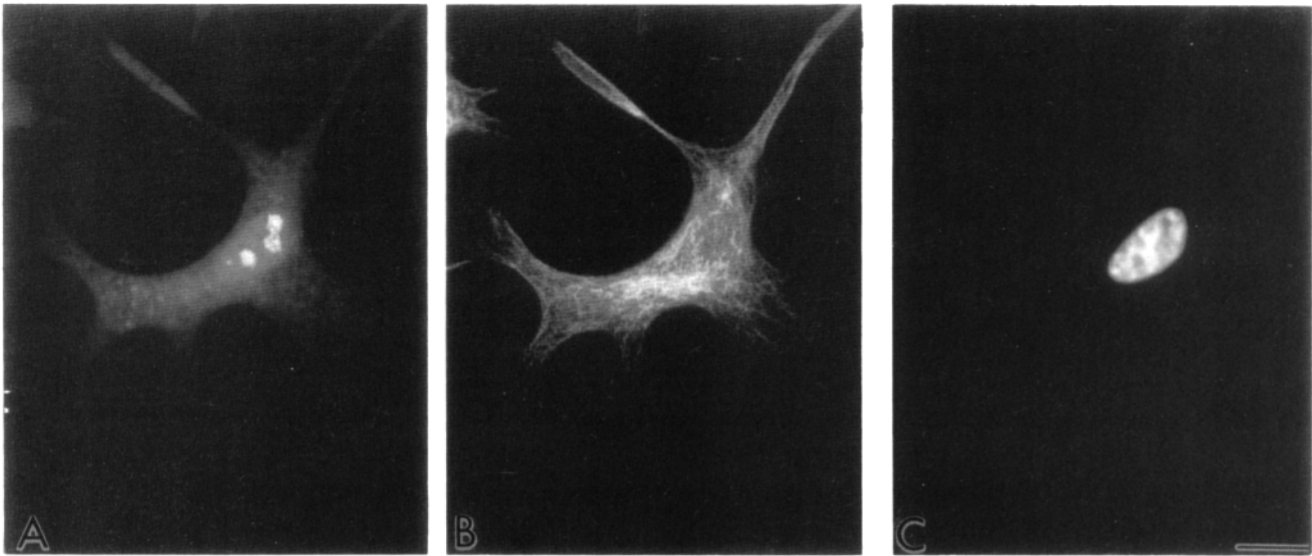
Figure 2. Double-label indirect immunofluorescence of a JC neuroblastoma cell stained with (*A*) Tau-1, (*B*) an anti-β–tubulin mAb (5H1) for microtubules, and (*C*) Hoechst 33258 for DNA. The cytoplasmic tau (*A*) does not colocalize with the microtubules (*B*) demonstrating that the cytoplasmic tau in this cell line, like the nuclear species is not microtubule-associated. Bar, 8 μm.

munofluorescence staining, however, all of the tau mAbs detected nuclear tau by immunoblot analysis (see Loomis et al., 1990).

Localization of Cytoplasmic Tau Using Indirect IF

Double IF experiments were performed using Tau-1 and an anti-β–tubulin mAb to determine the localization of the cytoplasmic tau in JC cells. The results of these experiments indicated that, in this cell line, cytoplasmic tau was not localized along microtubules, but rather was diffusely distributed in the cytoplasm (compare Fig. 2, *A* and *B*). Even when JC cells were permeabilized in microtubule stabilization buffer (to remove soluble cytoplasmic tau) before fixation, no microtubule associated tau was detected (data not shown).

Immunochemical Analysis of Tau in Cultured Cells

Nuclear and cytoplasmic fractions were prepared from CHO, CG and JC human neuroblastoma, and N_2A murine neuroblastoma cell lines. Since nuclear tau (like PHF tau) was found to be insoluble in SDS sample buffer it was necessary to extract the nuclear fractions with formic acid prior to desalting and reducing with Laemmli sample buffer (see Materials and Methods). Cytoplasmic tau was soluble in sample buffer and hence, the cytoplasmic fractions were extracted with sample buffer alone. Formic acid extraction of the cytoplasmic fraction was previously shown to be unnecessary to affect solubilization of cytoplasmic tau (Loomis et al., 1990).

Cytoplasmic and nuclear extracts were loaded into individual lanes and, after electrophoresis, were transferred to nitrocellulose and blotted against the mAb Tau-46.1. In all cases, the immunoblot analysis confirmed the presence of tau as detected by indirect immunofluorescence. CHO cell nuclei and cytoplasmic fractions were negative by the Tau-46.1 immunoblot assay (Fig. 3, *A* and *B*). Nuclear fractions from human neuroblastoma cell lines CG and JC were both positive for nuclear tau by immunoblot analysis (Fig. 3, *C*
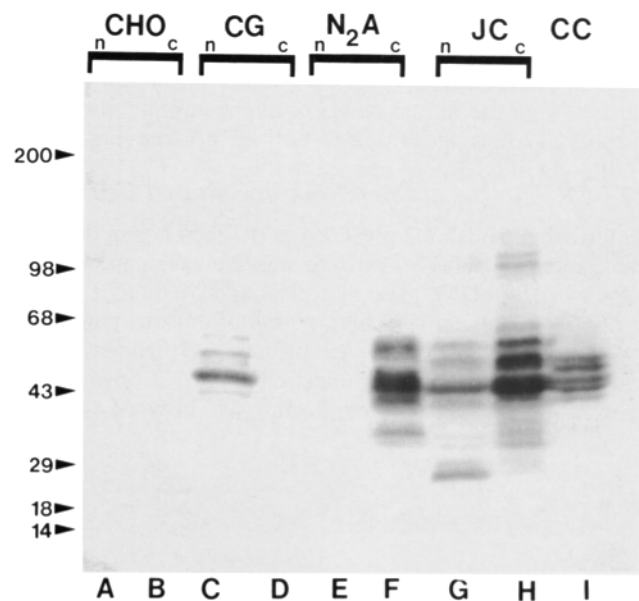

Figure 3. Western blot analysis of nuclear and cytoplasmic tau in various cell lines. Nuclear extracts (*n*) from CHO, CG, N_2A, and JC cells were isolated, extracted with 2.5% formic acid, and then solubilized with SDS. Cytoplasmic extracts (*c*) from the above cells were isolated and solubilized directly in SDS. Nuclear (lanes *A, C, E,* and *G* [75 μg/lane]) and cytoplasmic (lanes *B, D,* and *H* [50 μg/lane], and *F* [25 μg/lane]) fractions were then subjected to SDS-PAGE, transferred to nitrocellulose, and probed with the mAb Tau 46.1. Tau was absent from CHO cell extracts and was present in the nuclear fraction of CG and JC cells, and the cytoplasmic fraction of JC and N_2A cells. Immunoblots of nuclear and cytoplasmic tau exhibited the characteristic tau polypeptide pattern, similar to that observed in SDS extracts of human frontal cortex probed with Tau 46.1 (lane *I* [25 μg/lane]). Molecular weights are listed in kD.

and G) whereas, the nuclear fraction from N₂A murine neuroblastoma cells was negative by the Tau-46.1 immunoblot assay (Fig. 3 E). CG cells lacked Tau-1 immunostaining in their cytoplasm by IF and, as expected, no tau was detected in their cytoplasm by immunoblot assay (Fig. 3 D). In contrast, JC human and N₂A murine neuroblastoma cells both exhibited positive IF signals in their cytoplasm (Fig. 1, B′ and C′) and this was confirmed by immunoblot assay (Fig. 3, F and H). Additionally, tau polypeptides were readily detected in an extract from human frontal cortex (Fig. 3 I), as expected. The same results were obtained when the experiment was repeated and the immunoblots probed with the Tau-1 mAb (data not shown). The Tau-1 and Tau-46.1 mAbs recognize distinct epitopes located respectively in the central and carboxy-terminal regions of the tau molecule (Kosik et al., 1988).

Tau Northern Blot Analysis of Cultured Cells

The molecular nature of the message producing nuclear tau was explored by Northern blot analysis using a complete 3-repeat tau cDNA (see Materials and Methods). As expected, purified poly A⁺ mRNA from CHO cells, which lacked tau as assayed by IF and Western analysis, did not hybridize to the labeled probe (Fig. 4 A). The N₂A mouse neuroblastoma cells, which contained only cytoplasmic tau, exhibited hybridization to a major 6 kb and a minor 2 kb species (Fig. 4 C). Human CG neuroblastoma cells exhibited both a 6- and a prominent 2-kb hybridizing species (Fig. 4 B), while JC human neuroblastoma cells had only small amounts of the 6-kb tau transcript but instead displayed large amounts of 2-kb mRNA (Fig. 4 D). Interestingly, tau mRNA purified from the frontal cortex of a human brain also contained a 2-kb message as well as the 6-kb message (Fig. 4 E).

RT-PCR Analysis of Tau mRNA in Cultured Cells

To further establish the presence of tau message in the cell lines assayed, poly A⁺ mRNA was isolated and reverse transcribed to cDNA (see Materials and Methods). Using nested PCR, the entire coding region of tau was amplified for 30 cycles after which, new primers were added to the reaction to catalyze the amplification of the 515-bp encoded in exons 4–9. Secondary amplification within exons 4–9 was
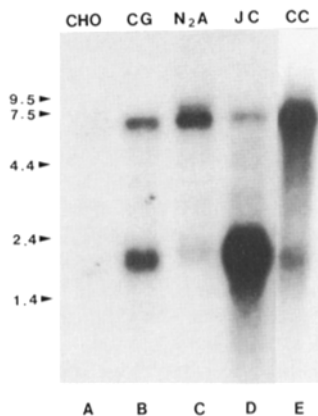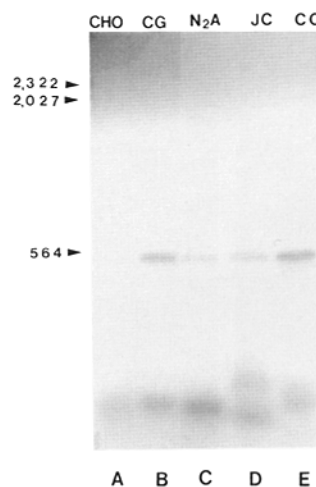
Figure 5. RT-PCR amplification of the tau mRNA from exons 4–9 in various cell lines. Poly A⁺ mRNA from CHO (lane A), CG (lane B), N₂A (lane C), and JC (lane D) cells, and from human frontal cortex (lane E) was isolated and subjected to reverse transcription. The resultant cDNA was then amplified for 30 cycles with tau primers (bases 307-327, 802-822). The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide and photographed. A video print was made from the negative, and hence, the reverse contrast. Reverse transcription and PCR amplification of mRNA from all samples except CHO (lane A) resulted in a tau cDNA product of 515 base pairs (lanes B–E). Molecular weight markers are given in base pairs.

chosen to determine whether exons 6 and/or 8 were present, since expression of these exons has not been reported in human tau although exon 6 has been found in mouse PNS tau (Couchie et al., 1992). Agarose gel electrophoresis of the resultant products of the PCR exhibited 515-bp cDNA bands in every cell line that had been shown to contain tau by IF and Western blotting. This size is consistent with a tau transcript lacking exons 6 and 8. Human CG and JC neuroblastoma cell lines both displayed an ethidium bromide-stained product (Fig. 5, B and D) as did mouse N₂A neuroblastoma cells (Fig. 5 C). RT-PCR revealed an identically sized product when poly A⁺ mRNA from human frontal cortex was used as a control (Fig. 5 E). Again, as expected, no RT-PCR product was observed when poly A⁺ mRNA from CHO cells was subjected to the same procedures (Fig. 5 A). These results further indicate that all of the cell lines shown to contain tau via IF, Western, and Northern blot analysis, including the CG neuroblastoma cells that contain only nuclear tau, express bona fide tau mRNA.

Analysis of the 2-kb mRNA in Cultured Cells

Both of the human neuroblastoma cell lines assayed contained an abundant 2-kb message in addition to the standard 6-kb tau message. Since a 2-kb tau transcript had not been reported previously, it was incumbent on us to prove that it constituted a genuine tau message. To accomplish this, poly A⁺ mRNA was isolated from human CG neuroblastoma cells that contained both the 6- and 2-kb messages. After electrophoresis on 1% low melting agarose denaturing gels, the 6- and 2-kb regions of the gel were excised, the mRNA extracted and subjected to RT-PCR using primers for the beginning of exon 1 and the middle of exon 9 (see Materials and Methods). The PCR products obtained from both the 2- (Fig. 6 A) and 6-kb (Fig. 6 C) messages were identical in size (528 bp). Similarly, PCR of a larger fragment (566 bp) was accomplished using primers beginning at nucleotide 669 of exon 9 and primers for the 3′ end of the coding region (see Materials and Methods). Again, this fragment was the same
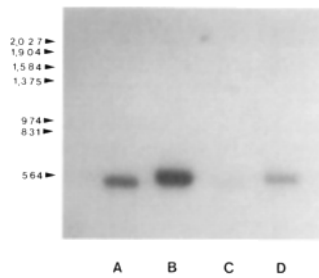
Figure 4. Northern blot analysis of poly A⁺ RNA isolated from various cell lines. 2 μg of poly A⁺ RNA from CHO (lane A), CG (lane B), N₂A (lane C), and JC (lane D) cells as well as poly A⁺ mRNA from human frontal cortex (lane E) was separated in a 1.4% agarose/formaldehyde gel, transferred to a nylon membrane, and probed with a ³²P-labeled human tau cDNA probe. Molecular weight markers on the left are given in kilobases. 2- and 6-kb transcripts were detected in mRNA isolated from human brain (lane E) and in the mRNA from all cell lines (lanes B–D) except CHO (lane A).

Figure 6. RT-PCR analysis of the entire coding region of human tau from both the 2- and 6-kb transcripts. Poly A$^+$ RNA from CG cells was isolated and fractionated on a 1% low melting point agarose/formaldehyde gel. The 2- and 6-kb mRNAs were excised from the gel, phenol/chloroform extracted, and subjected to reverse transcription. The resultant cDNAs were amplified for 30 cycles with the appropriate tau primers. RT-PCR products shown correspond to exons 1-9 of the 2-kb mRNA (lane *A*), exon 9 to the 3' end of the coding region of the 2-kb mRNA (lane *B*), exons 1-9 of the 6-kb mRNA (lane *C*), and exon 9 to the 3' end of the coding region of the 6-kb mRNA (lane *D*). RT-PCR data indicates the entire tau coding region is present in both the 2- and 6-kb mRNAs. Molecular weights listed on the left are in base pairs.

Figure 7. Origin of the 2-kb tau mRNA in human neuroblastoma cells. Cultures of CG human neuroblastoma cells were incubated with actinomycin D (5 μg/ml) for the times indicated below each lane. At the designated time points, poly A$^+$ RNA was extracted, separated by electrophoresis in a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled human cDNA probe. The 6-kb:2-kb ratio remained constant throughout the actinomycin D treatment, thus suggesting that the 2-kb mRNA is not derived from further processing of the 6-kb mRNA but rather from the original tau mRNA transcript.

size regardless of whether the PCR was performed from cDNA reverse transcribed from the 2- (Fig. 6 B) or the 6-kb (Fig. 6 D) mRNA. These results strongly suggest that the entire coding region of tau is present in the 2- as well as the 6-kb message. Subsequent cDNA sequencing of both of the 2-kb PCR products (Fig. 6, *A* and *B*) was performed after insertion of each product into sequencing vectors (see Materials and Methods). The sequence obtained represented the entire coding region of the smallest human 3-repeat tau isoform (i.e., lacking exons 2, 3, and 10) as described by Goedert et al. (1989) (data not shown).

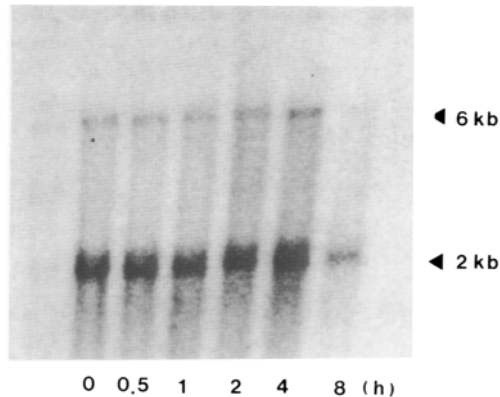Since the 2-kb message clearly contains the complete coding region of tau, experiments were performed to ascertain whether the 2-kb species was derived from the 6-kb mRNA or from the original tau transcript. Cultures of CG human neuroblastoma cells were incubated with actinomycin D (5 μg/ml) for 0, 0.5, 1.0, 2.0, 4.0, and 8 h. At each time point, poly A$^+$ RNA was extracted and 5 μg/lane was loaded onto a denaturing 1% agarose gel, and separated by electrophoresis. The resultant gel was transferred to a nylon membrane and probed with a labeled human tau cDNA probe. Subse-
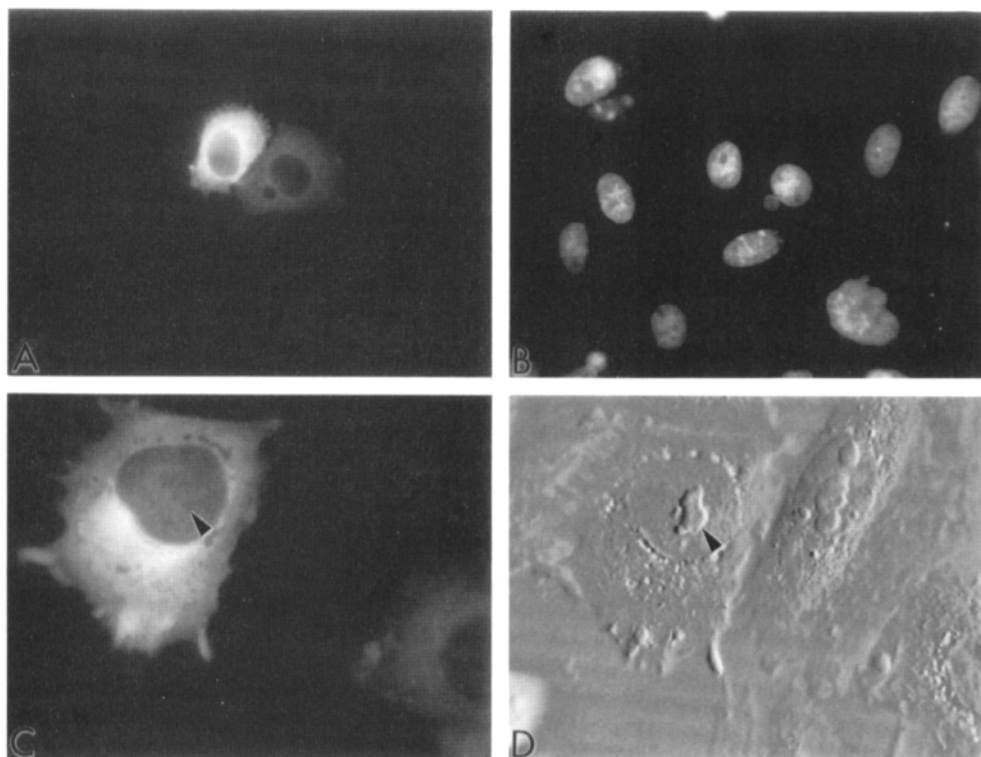

Figure 8. Transient transfection of the whole coding region of three repeat tau into CHO cells. CHO cells which have been shown not to express tau (see above) were plated on coverslips and transfected with the pEn123c eukaryotic expression vector containing the coding region of three-repeat short human tau (see Materials and Methods). The cells were cultured after transfection for an additional 42 h before fixation and processing for IF using our Tau-1 monoclonal antibody. (*A*) Tau-1 immunostaining; (*B*) Hoechst 33258 DNA staining; (*C*) Higher magnification of a transfected cell and (*D*) Differential interference contrast microscopy of the same field. The arrowheads in *C* and *D* denote the position of the nucleoli in the transfected cells. Tau-1 IF staining was never detected in nucleoli of these transiently transfected cells.

quent autoradiography demonstrated that the 6-kb:2-kb ratio remained roughly constant at each time point indicating that the 2-kb message does not arise from further processing of the 6-kb mRNA (Fig. 7). This data suggests that the 2-kb message is generated from the original tau transcript by alternative splicing, usage of alternative polyadenylation signals, or both.

Transient Transfection of Tau Coding Region into CHO Cells

The presence of tau in the nucleus was correlated with an abundant 2-kb tau transcript. This suggested that the untranslated region of the 2-kb mRNA may be playing a role in targeting tau for nuclear import. This argument would be moot, however, if transfection of the coding region of the tau transcript produced tau that localized in the nucleolus. Since we demonstrated by cDNA sequencing of RT–PCR products that at least the entire coding region of the three-repeat, short tau mRNA was present in the 2-kb transcript, a transfection experiment was performed in CHO cells. CHO cells were chosen since they lacked tau as determined by IF, Northern blotting, PCR, and immunoblot assays (see above). After transfection as outlined in Materials and Methods, combined DIC and Tau-1 IF analyses demonstrated that, although the transfections clearly were successful, nucleolar staining was never observed (Fig. 8, A–D). Identical experiments were also performed using a four-repeat-long tau cDNA (containing exons 2, 3, and 10); these yielded identical results (data not shown).

Discussion

Nuclear Tau and AD

Tau proteins are now known to comprise an integral portion of the PHFs that aggregate to form the neurofibrillary tangles in vulnerable neurons in AD brains. Two classes of PHFs can be isolated from AD brain, one readily soluble in SDS and an insoluble PHF form that often resists extraction with SDS (Ihara et al., 1983; Iqbal et al., 1984; Wischik et al., 1988). Nuclear tau also withstands extraction with SDS much like the insoluble form of PHF, requiring harsh chemical treatments for solubilization. Immunohistochemical and immunochemical studies have demonstrated that both forms of PHF tau are abnormally phosphorylated at several sites (Grunke-Iqbal et al., 1986a,b; Ksiezak-Reding et al., 1990; Greenberg et al., 1992; Goedert et al., 1992a) while the phosphorylation state of nuclear tau is unknown largely because the means used to extract it (formic acid) removes phosphate groups from proteins. Nonetheless, our data establishes the existence of a highly insoluble form of tau as a normal component of human cell nuclei compelling further investigation into the relationship between PHF formation during AD pathogenesis and nuclear tau.

Aside from having solubility properties in common with PHF, nuclear tau is also found on the short arms of the human acrocentric chromosomes (# 13, 14, 15, 21, and 22) at the NOR regions (Loomis et al., 1990). These chromosomes are prone to nondisjunction resulting in trisomy and many investigators suggest that this is due to aggregation and cohe-

siveness at their NOR regions during metaphase (Markovic et al., 1978; Hansson, 1979; Mirre et al., 1980; Jackson-Cook et al., 1985; Jones et al., 1988). Of acrocentric chromosome trisomies, only trisomy 21 (Down Syndrome) results in viable offspring and most of these patients develop AD if they live into their fourth and fifth decades (Wisniewski et al., 1985). Furthermore, in the inherited form of AD, Familial AD, families exhibit a higher incidence of trisomy 21 offspring (Heston et al., 1981; Heyman et al., 1983a,b; Heston, 1984; Cutler et al., 1985; Potter, 1991), again indicating a connection between AD and Down Syndrome. Our work places the PHF protein, tau, at the putative site of nondisjunction leading to Down Syndrome, and eventual AD. Although the evidence is circumstantial, tau must now be considered as a candidate for the molecular link between these two disease states.

Significance of the 2-kb mRNA

Before the work described herein, tau was thought to be encoded by either a 6- or an 8-kb message. The 6-kb message is found in abundance only in the central nervous system (Drubin et al., 1984; Neve et al., 1986; Kosik et al., 1989b). The 8-kb mRNA, on the other hand, is mostly restricted to the peripheral nervous system, and, in rats and mice, contains an extra exon inserted between exons 4 and 5 resulting in a markedly larger tau molecule (Goedert et al., 1992b; Couchie et al., 1992). A 1.7-kb tau message has recently been reported as the only tau transcript in testes (Ashman, et al., 1992); however, in the CNS, where tau is most often studied, only a 6-kb transcript has been described. Examination of a recent paper (Goedert et al., 1988) reveals a 2-kb hybridizing species in Northern blots from human frontal cortex. Interestingly, this paper demonstrated that the 6-kb tau message was not upregulated in AD brain; however, in the same figure, an upregulated 2-kb message was quite apparent. Here, we demonstrate that the 2-kb message is the prominent tau mRNA in two human neuroblastoma cell lines and confirm its presence in human brain. Thus far, we have been able to detect only a relatively small amount of 2-kb message in Northern blots of murine neuroblastoma mRNAs.

The molecular nature of the 2-kb mRNA is interesting in that, although it appears to originate from the original tau transcript rather than resulting from further processing of the 6-kb mRNA, the 2-kb species apparently contains the entire coding region of human tau as determined by RT–PCR sizing gels and cDNA sequencing. This indicates that the differences between the 6- and 2-kb transcripts reside in the untranslated regions (UTR) with the size differential between these two mRNAs being generated by alternate splicing within the UTR, multiple polyadenylation sites, or both. A targeting role for the UTR has been recently postulated for numerous mRNAs (MacDonald et al., 1988; Singer, 1992) and hence, it is possible that the differences between the 2- and 6-kb transcripts may reflect a differential targeting of tau catalyzed by the UTR. In support of this, it is noteworthy that, although JC human neuroblastoma were shown to contain both nuclear and cytoplasmic tau, localization of tau along microtubules was not observed. These cells contained very little 6-kb transcript but instead displayed an abundant 2-kb mRNA hybridizing species. From this result, it is possible that the UTR of the 2-kb mRNA in humans may serve

to target the message to regions of the cytoplasm specifying both tau translation and posttranslational modifications specific for nuclear and nonmicrotubule cytoplasmic functions.

Cytoplasmic Non-microtubule Tau Associations

Previously, we demonstrated that tau localized not only along microtubules within the axonal and somatodendritic compartments of rat neurons in situ but also localized on ribosomes (Papasozomenos and Binder, 1987). The functional significance of this observation is still unclear; however, its importance is underscored by reports of abnormal tau associated with ribosomes in both astrocytes and neurons in AD brain (Papasozomenos, 1989). One possible function for non-microtubule, ribosome-associated tau may be to target these organelles to microtubules for transport into the somatodendritic compartment of neurons to facilitate local protein synthesis. The molecular mechanisms responsible for targeting tau to ribosomes vs. microtubules within the neuron are unknown. Above we suggested that this may be accomplished at the mRNA level in human cells: the 2-kb message targeting to non-microtubule functions such as ribosome binding and the 6-kb message specifying targeting for direct microtubule binding. However, it should be noted that IF analysis of N_2A mouse neuroblastoma cells also indicated the presence of predominantly nonmicrotubule cytoplasmic tau which, according to the accompanying Northern blot determinations, would have been transcribed from a 6-kb message. Clearly then, in this rodent cell line, the 6-kb message must have the ability to target to both microtubule and non-microtubule regions of the cell. Therefore, further insight into the precise targeting mechanisms for different functional tau associations awaits sequencing of the UTRs of both human and rodent 2- and 6-kb messages.

The Transport of Tau into the Nucleus

The current report and previous studies (Loomis et al., 1990) firmly establish the existence of nuclear tau, although the mechanism by which it transits into the nucleus from the cytoplasm remains to be determined. Although there does not appear to be a strict consensus sequence for nuclear import (reviewed in Garcia-Bustos, 1991), most import signals are short sequences of approximately 8–10 basic amino acids that can be located anywhere within the protein. It may be possible that the basic three or four repeat microtubule binding domains are sufficient to catalyze tau's entrance into the nucleus. However, the transient transfection experiments described above indicate that expression of large amounts of tau alone is not sufficient to induce its transit into the nucleolus in CHO cells.

Another possibility is that tau is entering into the nucleus by interacting with other nuclear proteins containing a nuclear import sequence. Apparently, nuclear proteins lacking nuclear import signals can "piggyback" into the nucleus by interacting with a nuclear protein containing this sequence (Moreland et al., 1987; Zhao et al., 1988; Booher et al., 1989). Hence, determination of the means facilitating tau's entry into the nucleus may further lengthen the list of proteins interacting with this multifunctional MAP.

Nuclear Tau Isoforms

The electrophoretic patterns observed in formic acid–treated extracts of nuclear tau indicate that many, if not all, of the six tau isoforms are present within the nucleus even though the sequence obtained from the RT-PCR product of the coding region of the 2-kb message was that of three-repeat tau lacking amino terminal exons 2 and 3. However, PCR of multiple transcripts would intuitively be expected to enrich for the shortest cDNA encoding the shortest isoform, and this was apparently the case. Other isoforms are amplified to a lesser extent by PCR, and although they are not observed in an agarose/ethidium bromide gel, they can be detected on Southern blots (data not shown).

Significance of Nuclear Tau

The results presented here and elsewhere (Seldon and Pollard, 1983; Kotani et al., 1985; Miyata et al., 1986; Papasozomenos and Binder, 1987; Jancsik et al., 1989; Rendon et al., 1990; Loomis et al., 1990) establish tau as a multifunctional protein. Although the function of nuclear tau is a matter of speculation, since no microtubules exist within the nucleus, an alternative raison d'être for tau is warranted. As mentioned above, we reported that tau was found not only in axons of neurons but also in the somatodendritic compartments where it was localized not only on the microtubules but also, on the ribosomes (Papasozomenos and Binder, 1987). This, together with our previous report (Loomis et al., 1990) suggests one of tau's alternate functions may involve some aspect of protein synthesis since it is not only associated with ribosomes but also with the NOR regions of the acrocentric chromosomes, the sites of the rRNA genes, and the fibrillar regions of the nucleoli, the sites of rRNA transcription. The role of nuclear tau in protein synthesis, however, is further shrouded by its absence in nonprimate cell lines indicating that this new function for tau is a recent evolutionary event. As yet, the selective advantages that nuclear tau bestows upon primate NORs or rRNA transcription are unknown.

Perhaps some clues as to the function of nuclear tau can be derived by knowing where it is not. Nuclear tau has not been reported in primate neurons or glia, in situ or in culture. We have documented its existence in dividing cells in culture and perhaps this indicates that nuclear tau disappears upon terminal differentiation in the nervous system. The localization in dividing cells of nuclear tau on the NORs during mitosis and within the fibrillar regions of the nucleolus at interphase may establish a role for tau in initiating nucleolar reformation and/or mediating the onset of rRNA synthesis. Once cells are arrested in G1 and are differentiated, the functions mediated by tau may no longer be needed and hence, nuclear tau synthesis and/or import into the nucleus would simply cease.

In summary, this work establishes tau as much more than a neuronal MAP and further suggests message targeting as a mechanism of controlling tau's functional interactions. However, the precise relationship of the 2-kb message to nuclear and nonmicrotubule tau associations awaits the discovery of either a cell line that expresses only 2-kb tau or the transfection of a cDNA encoding the entire 2-kb message into a cell that expresses neither 6- nor 2-kb tau. Clearly, transfecting the entire coding region of tau into CHO cells was not sufficient to induce tau movement into the nucleus and nucleolus. A more definitive transfection experiment mandates the sequencing of the UTRs of the 2-kb tau

mRNAs facilitating transfection of the entire transcript; these experiments are currently underway.

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