Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8422–8426, November 1990
Cell Biology

Identification of nuclear τ isoforms in human neuroblastoma cells

(nucleolus/microtubule-associated proteins/Alzheimer disease/Down syndrome)

P. A. LOOMIS*†, T. H. HOWARD‡, R. P. CASTLEBERRY‡, AND L. I. BINDER*

Departments of *Cell Biology and ‡Pediatrics, University of Alabama at Birmingham, Birmingham, AL 35294

Communicated by Joseph G. Gall, August 9, 1990 (received for review June 25, 1990)

ABSTRACT **The τ proteins have been reported only in association with microtubules and with ribosomes *in situ*, in the normal central nervous system. In addition, τ has been shown to be an integral component of paired helical filaments, the principal constituent of the neurofibrillary tangles found in brains of patients with Alzheimer disease and of most aged individuals with Down syndrome (trisomy 21). We report here the localization of the well-characterized Tau-1 monoclonal antibody to the nucleolar organizer regions of the acrocentric chromosomes and to their interphase counterpart, the fibrillar component of the nucleolus, in human neuroblastoma cells. Similar localization to the nucleolar organizer regions was also observed in other human cell lines and in one monkey kidney cell line but was not seen in non-primate species. Immunochemically, we further demonstrate the existence of the entire τ molecule in the isolated nuclei of neuroblastoma cells. Nuclear τ proteins, like the τ proteins of the paired helical filaments, cannot be extracted in standard SDS-containing electrophoresis sample buffer but require pretreatment with formic acid prior to immunoblot analysis. This work indicates that τ may function in processes not directly associated with microtubules and that highly insoluble complexes of τ may also play a role in normal cellular physiology.**

τ proteins were first identified as a family of phosphoproteins that associate with microtubules *in vivo* and stimulate their assembly *in vitro* (1, 2). Recent evidence indicates that τ proteins are an integral component of the paired helical filaments, the principal constituent of the neurofibrillary tangles characteristic of Alzheimer disease or senile dementia of the Alzheimer type (3–7). The structure of the τ gene and of the cDNAs cloned and sequenced from the expressed mRNAs reveals a tripartite protein composed of a variable N-terminal domain, a constant central domain, and a C-terminal, tubulin-binding domain (8, 9). Hence, it appears that much of the observed electrophoretic heterogeneity is generated by alternative splicing of a single RNA transcript (8–10).

Initially, τ was reported to be restricted to axons within the central nervous system (11). A more widespread distribution was subsequently documented, indicating the presence of τ along microtubules of both the axonal and somatodendritic compartments (12). Additionally, τ was observed on ribosomes in neuronal somatodendritic compartments and in glial cells (12). We report here the localization of the Tau-1 monoclonal antibody (11) to the nucleolar organizer regions (NORs) of the acrocentric chromosomes (nos. 13, 14, 15, 21, and 22), in cultured human cells. Immunolocalization is also detected in the interphase counterpart of the NORs, the fibrillar component of nucleoli. Similar localization patterns are observed in cultured monkey kidney cells but are not present in non-primate cultured cell lines. The presence of τ is biochemically documented in the isolated nuclei of two human neuroblastoma cell lines. These results suggest that τ is involved in some aspect of nucleolar structure and/or function in primates. Much of this work has been presented in abstract form (13).

MATERIALS AND METHODS

Cell Culture. The cell lines JC and CG (human neuroblastoma cells), CV-1 (African green monkey kidney cells), WI-38 (human lung fibroblasts), HeLa (human cervical carcinoma cells), BHK-21 (baby Syrian hamster kidney cells), and NA2 (mouse neuroblastoma cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human macrophages and WERI (human retinoblastoma cells) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Muntjak (Indian muntjak deer skin cells) and PtK_2 (rat kangaroo kidney cells) were cultured in Ham's F-12 medium supplemented with 15% fetal bovine serum. CHO (Chinese hamster ovary cells) were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum. All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Immunofluorescence. Cells grown on glass coverslips were fixed in 3% formaldehyde (Tousimis) for 30 min at 24°C. Isolated chromosomes centrifuged onto glass coverslips were fixed in 75% ethanol for 30 min at −20°C. Both cell and chromosome preparations were then processed for indirect immunofluorescence microscopy (14). Chromosomes were visualized with propidium iodide. The processed coverslips were mounted onto glass slides and then viewed and photographed on a Leitz photomicroscope.

Isolation and Extraction of Nuclei. Nuclear and cytoplasmic fractions from human neuroblastoma cells were isolated as described by Mitchison and Kirschner (15). The pelleted nuclei were washed and resuspended in 100 mM Tris (pH 6.8), sonicated for 15 sec, and either solubilized by boiling in electrophoresis sample buffer (62.5 mM Tris, pH 6.8/2% SDS/10% glycerol/5% 2-mercaptoethanol; ref. 16) or extracted with 10% formic acid, desalted, and solubilized by boiling in electrophoresis sample buffer. Cytoplasmic fractions were concentrated using an Amicon ultrafiltration device (Diaflo, YM10 filter) prior to treatment with electrophoresis sample buffer as described above.

Gel Electrophoresis. Protein concentrations were determined by a modification of the method of Lowry *et al.* (17) after precipitation with 10 volumes of 10% perchloric acid/1% phosphotungstic acid. Samples were electrophoresed in SDS/5–12.5% linear polyacrylamide gradient gels (16), and the separated proteins were transferred to nitrocellulose (18) and probed with the τ monoclonal antibodies Tau-1 (11), Tau-46.1 (19), Tau-60 (19), and 5E2 (19–21). Bound antibodies were detected using peroxidase-conjugated secondary antibody (11).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: NOR, nucleolar organizer region.
†To whom reprint requests should be addressed.

RESULTS

Distribution of Tau-1 Immunoreactivity During Mitosis. When human neuroblastoma cells (line CG) were examined by indirect immunofluorescence using the Tau-1 monoclonal antibody, nucleoli were the only structures labeled (Fig. 1*a*). Parallel silver staining confirmed that the intense punctate staining colocalized with the active fibrillar components of the nucleoli (data not shown) (22–27). Upon entering prometaphase, Tau-1 localization was observed as 8–10 spherical structures associated with certain chromosomes (Fig. 1*b*). Similar chromosomal localization was detected throughout metaphase and anaphase (Fig. 1 *c* and *d*). Tau-1 reactivity in telophase was coincident with the prenucleolar bodies of the re-forming nucleolus (Fig. 1*e*), presumably due to their association with the NORs (28, 29).

The localization of Tau-1 to the NORs was confirmed by examining metaphase spreads of isolated chromosomes. In all cases, Tau-1 localized to the short arms of the acrocentric chromosomes (nos. 13, 14, 15, 21, and 22), the well-documented location of human NORs (30, 31) (Fig. 2). Similar Tau-1 localization patterns were not restricted to one neuroblastoma cell line but were also observed in the nucleoli of monkey and several human cell lines (Table 1). However, although the Tau-1 monoclonal antibody reacts with brain τ of species ranging from *Xenopus* (32) to humans (4, 7), no nucleolar localization was observed in cells from nonprimates.

Synthetic Peptide Absorption Analysis. Since Tau-1 was the only monoclonal antibody that displayed nucleolar staining, there was concern that we were observing a nonspecific interaction. Therefore, a synthetic peptide encompassing the Tau-1 epitope (19) was constructed and used for absorption analysis. When coincubated with the Tau-1 monoclonal antibody, this 21-amino acid peptide completely inhibited nucleolar staining in human neuroblastoma cells, thus firmly

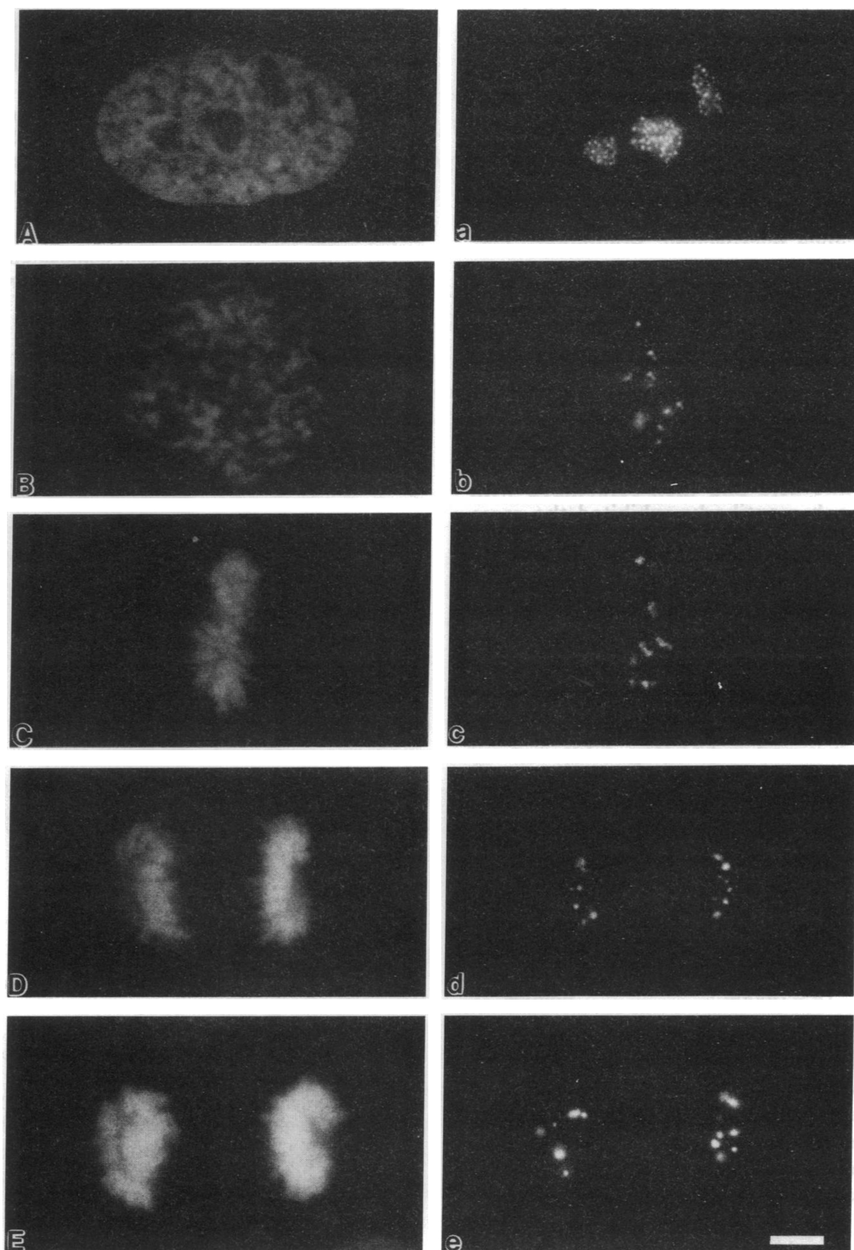

FIG. 1. Localization of Tau-1 immunoreactivity during mitosis of CG human neuroblastoma cells. Each cell shown was stained for DNA with Hoechst 33358 (*A–E*), and for τ protein reactivity by indirect immunofluorescence (*a–e*). (*A* and *a*) Interphase. (*B* and *b*) Prometaphase. (*C* and *c*) Metaphase. (*D* and *d*) Anaphase. (E and *e*) Telophase. (Bar = 2 μm.)

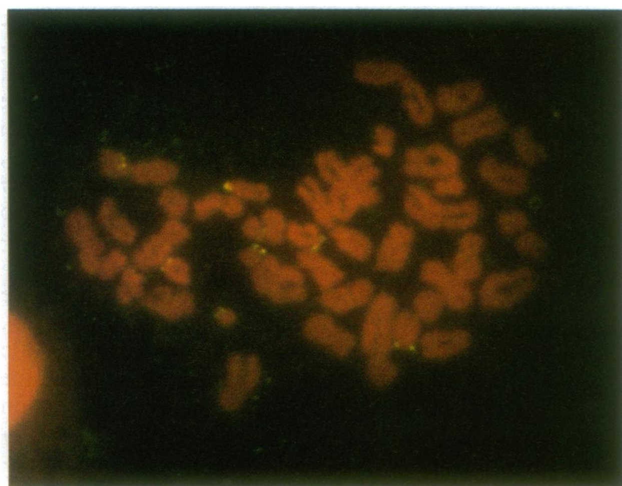

FIG. 2. Localization of Tau-1 immunoreactivity on isolated neuroblastoma (line CG) chromosomes. Chromosomes were stained for DNA with propidium iodide and for τ protein reactivity by indirect immunofluorescence.

establishing the presence of the Tau-1 epitope in the nucleolus (Fig. 3). Since detection of the Tau-1 epitope can be phosphatase-dependent (4, 12), we subjected human neuroblastoma cells to dephosphorylation prior to analysis by indirect immunofluorescence microscopy. Preliminary evidence indicates that the Tau-1 staining was unaffected by phosphatase (data not shown).

Immunoblot Analysis. Human neuroblastoma nuclei were examined biochemically for the presence of additional τ protein epitopes. Immunoblots of isolated nuclei solubilized in conventional SDS electrophoresis buffer and probed with a panel of τ antibodies were consistently negative (Fig. 4*A*, lanes 1, 3, 5, and 7). However, if the nuclei were first extracted with 10% formic acid and then solubilized with SDS, immunoblots of each τ antibody exhibited the same characteristic τ protein pattern (lanes 2, 4, 6, and 8), similar to that observed in SDS extracts of human cerebral cortex (lane 9). Furthermore, the antibodies used bind to sequences on τ that nearly span the molecule (Fig. 4*B* and ref. 19), indicating that the complete τ molecule is present in the nuclei of human neuroblastoma cells.

When the human neuroblastoma cell lines CG and JC were examined by indirect immunofluorescence microscopy, only nucleolar staining was observed in CG (Fig. 5*A1*), but nuclear, nucleolar, and diffuse cytoplasmic labeling were seen in JC (Fig. 5*A2*). A microtubule staining pattern was never observed following Tau-1 immunostaining, even though under the fixation conditions utilized, tubulin antibodies local-

Table 1. Indirect immunofluorescence detection of nucleolar τ in cultured cells

Cell line	Description	Tau-1 epitope
BHK-21	Baby Syrian hamster kidney	−
CG	Human neuroblastoma	+
CHO	Chinese hamster ovary	−
CV-1	African green monkey kidney	+
HeLa	Human cervical carcinoma	+
JC	Human neuroblastoma	+
—	Human macrophages	+
Muntjak	Indian muntjak deer skin	−
NA2	Mouse neuroblastoma	−
PtK_2	Rat kangaroo kidney	−
WERI	Human retinoblastoma	+
WI-38	Human lung fibroblast	+

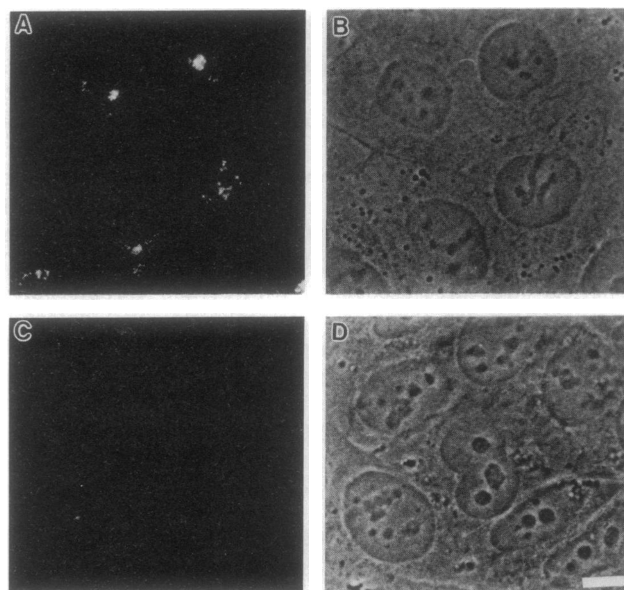

FIG. 3. Absorption of Tau-1 immunostaining by a synthetic peptide (PKSGDRSGYSSPGSPGTPGSR) that encompasses the Tau-1 epitope (19). (*A*) Cells were incubated with Tau-1 (1:80 dilution) followed by a fluorescein-conjugated secondary antibody. (*B*) Phase-contrast image of cells in *A*. (*C*) Cells were incubated with Tau-1 (1:80 dilution) plus the synthetic peptide (15 ng/ml) described above, followed by fluorescein-conjugated secondary antibody. (*D*) Phase-contrast image of cells in *C*. (Bar = 5 μm.)

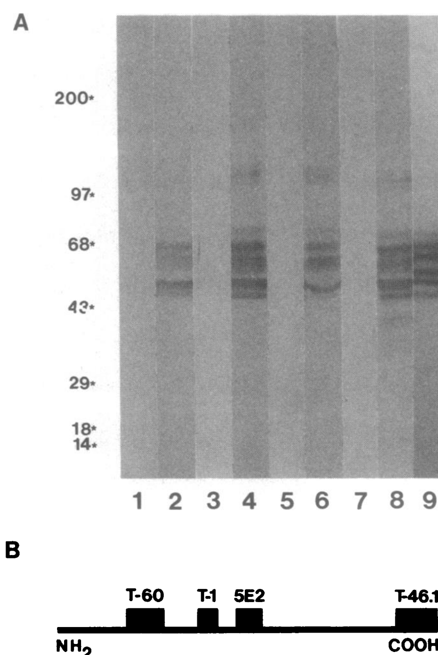

FIG. 4. Epitope mapping of nuclear τ. (*A*) Nuclei (50 μg of protein per lane) were solubilized with SDS (lanes 1, 3, 5, and 7) or extracted with formic acid and solubilized with SDS (lanes 2, 4, 6, and 8). Proteins were separated in SDS/5–12.5% linear polyacrylamide gradient gels and transferred to nitrocellulose, and the blots were probed with the monoclonal antibodies Tau-60 (lanes 1 and 2), Tau-1 (lanes 3 and 4), 5E2 (lanes 5 and 6), and Tau-46.1 (lanes 7 and 8). Immunoblots of each τ antibody exhibited the same characteristic τ protein pattern, similar to that observed in SDS extracts of human cerebral cortex probed with Tau-1 (lane 9). Molecular size markers (kDa) are at left. (*B*) Positions of the epitopes recognized by these four monoclonal antibodies on fetal human τ (19) are shown schematically. T, Tau.

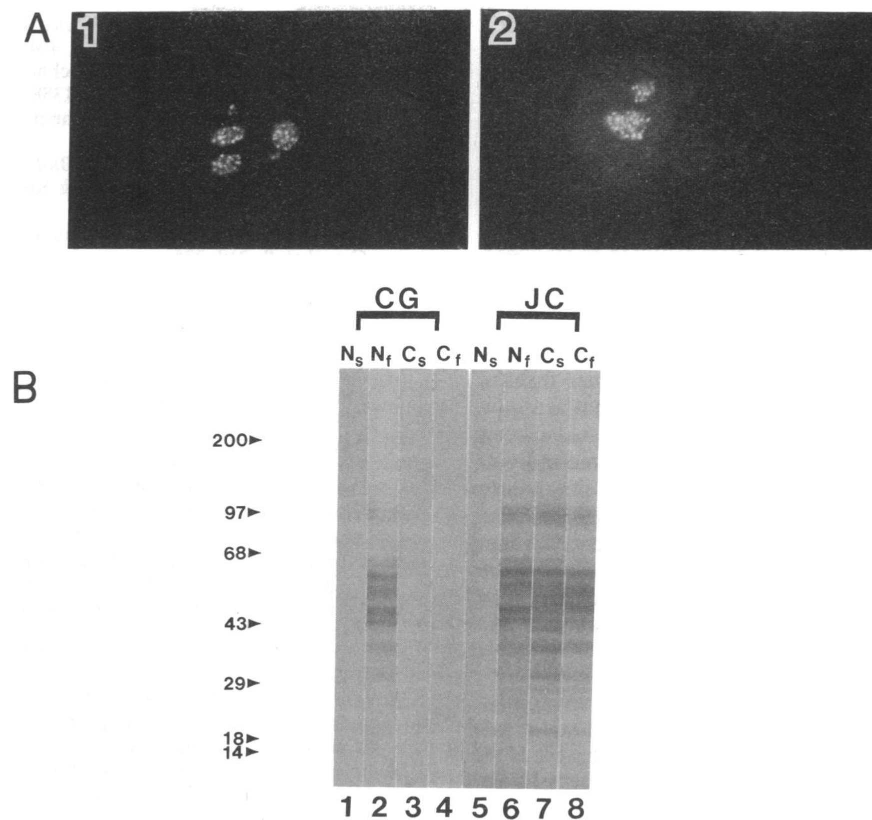

FIG. 5. (*A*) Immunolocalization of Tau-1 in human neuroblastoma cell lines CG (*1*), and JC (*2*). CG cells display nucleolar staining, while JC cells display both nucleolar and diffuse cytoplasmic labeling. (*B*) Immunoblots of isolated nuclei and cytoplasm from human neuroblastoma cell lines (CG and JC) probed with the Tau-1 monoclonal antibody. Nuclear (lanes 1, 2, 5, and 6) and cytoplasmic (lanes 3, 4, 7, and 8) fractions were isolated and solubilized with SDS (lanes 1, 3, 5, and 7) or extracted with 10% formic acid and solubilized with SDS (lanes 2, 4, 6, and 8). CG samples (75 μg, lanes 1–4) and JC samples (35 μg, lanes 5–8) were subjected to SDS/polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with Tau-1. N_s, nuclear fraction solubilized with SDS; C_s, cytoplasmic fraction solubilized with SDS; N_f, nuclear fraction extracted with formic acid prior to solubilization with SDS; C_f, cytoplasmic fraction extracted with formic acid prior to solubilization with SDS.

ized in the classic fibrillar array (data not shown). In fact, using numerous τ antibodies, we have never documented a microtubule localization pattern in these two cell lines.

Nuclear and cytoplasmic fractions isolated from the two human neuroblastoma cell lines were solubilized with SDS or extracted with 10% formic acid and then solubilized with SDS. Immunoblots were probed with Tau-1. Even in the cell line that contains cytoplasmic τ (line JC), no τ was detected in the nuclear fraction (Fig. 5*B*, lane 5) unless the sample was first extracted with formic acid (lane 6). Moreover, the cytoplasmic τ signal was not increased by formic acid extraction (lane 8), indicating that SDS-insoluble τ is present only in the nucleus. Thus, the nucleolar staining pattern is consistent in human neuroblastoma cell lines that do (JC) and do not (CG) biochemically exhibit τ protein reactivity in the cytoplasm.

DISCUSSION

These results conclusively demonstrate the existence of SDS-insoluble τ in the nucleus of human neuroblastoma cells and further indicate that at least some of this τ is localized in the nucleolus. Thus far, only the Tau-1 antibody localizes to the nucleolus as judged from indirect immunofluorescence. Why only one τ monoclonal antibody displays this localization pattern is not known, and it is possible that the nucleolar protein binding the Tau-1 antibody is not τ but merely shares the Tau-1 epitope. We believe this is unlikely, since our immunoblot analysis indicates that Tau-1 recognizes only τ and since three additional τ monoclonal antibodies, whose epitopes span the molecule, react with these same polypeptides present in purified nuclei.

The presence of τ protein epitopes in the nuclei of human and monkey cell lines suggests an expanded role for this protein in normal cellular physiology. The localization of Tau-1 to the NORs and the fibrillar component of the nucleolus is of particular significance since these regions contain the rRNA genes. Perhaps of more interest is that our preliminary evidence indicates that Tau-1 staining colocalizes with the active fibrillar regions identified by silver staining (22–27). Active fibrillar regions are those actually involved in rRNA transcription; only these regions stain with silver and, apparently, with the Tau-1 monoclonal antibody (P.A.L. and L.I.B., unpublished observation). This result is even more intriguing when one considers that the only other non-microtubule localization reported to date indicated the presence of τ proteins on ribosomes in neurons and glia (12). Hence it appears that τ is, in some way, involved in ribosome biology. Whether this involvement is related to ribosome synthesis, assembly, structure, transport, or some other process is unknown.

While the function of nuclear τ is unknown, an examination of this τ protein's special characteristics may help to elucidate its interactions with the macromolecules of the nucleolus. Analysis of the τ gene by Himmler (10) indicated the existence of previously unreported τ isoforms with C-terminal extensions containing multiple cysteine and histadine residues. These extensions reportedly resemble metal-binding finger domains, raising the possibility that certain τ protein isoforms may be DNA-binding proteins (33). Con-

ceivably, these sequences could mediate τ protein interactions with the rRNA genes of the NORs and the RNA molecules of ribosomes in mature neurons (12).

An additional special characteristic of nuclear τ is its insolubility in SDS sample buffer prior to extraction with formic acid. Altered extractability is a property nuclear τ proteins share with the τ found in paired helical filaments (PHFs) (34). PHFs are the main constituent of the neurofibrillary tangles, the diagnostic pathological features of damaged neurons in Alzheimer disease (35–37). Thus, highly insoluble complexes of τ also appear to be involved in normal cellular functions.

Investigation of nuclear τ proteins may advance our understanding of the relationship between Alzheimer disease and Down syndrome. Neurofibrillary changes that are now known to involve incorporation of τ into PHFs have been documented in the brains of some Down syndrome individuals during adolescence. By the fourth decade of life, nearly all Down syndrome individuals are demented (38). The presence of a form of τ on the acrocentric chromosomes that has unusual extractability properties like the τ present in the PHFs of neurofibrillary tangles suggests that nuclear τ may be involved in a common pathogenesis linking trisomy 21 and Alzheimer disease.

We thank Dr. Claudia Caputo (ICI Pharmaceuticals, Wilmington, DE) for her gift of the synthetic peptide used in these studies; Dr. Ken Kosik (Brigham and Women's Hospital, Harvard) for his gift of the antibody 5E2; and Dr. Virginia Lee (University of Pennsylvania, Department of Pathology and Laboratory Medicine) for providing the Tau 46.1 and Tau 60 antibodies. We wish to acknowledge the invaluable assistance of C. Davis, K. Brown, and A. Tousson. We are grateful to Dr. H. Kim and Dr. B. R. Brinkley for critically reviewing the manuscript. Finally, we thank R. Zinkowski for incisive discussions and for sharing his technical prowess with us, allowing the accomplishment of these experiments. This work was supported by National Institutes of Health Grants AG06969 (to L.I.B.), AI25214 (to T.H.H.), and CA25408 (to R.P.C.).

1. Connolly, J. A., Kanins, V. I., Cleveland, D. W. & Kirschner, M. W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2437–2440.
2. Weingarten, M. D., Lockwood, A. H., Hwo, S. Y. & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1858–1862.
3. Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, T.-C., Zaidi, M. S. & Wisniewski, H. M. (1986) *J. Biol. Chem.* **261**, 6084–6089.
4. Grundke-Iqbal, I., Iqbal, K., Tung, T.-C., Quinlan, M., Wisniewski, H. M. & Binder, L. I. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4913–4917.
5. Ihara, Y., Nikina, N., Miura, R. & Ogawara, M. (1986) *J. Biochem (Tokyo)* **99**, 1807–1810.
6. Kosik, K. S., Joachim, C. & Selkoe, D. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4044–4048.
7. Wood, J. G., Mirra, S. S., Pollock, N. J. & Binder, L. I. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4040–4043.
8. Himmler, A., Drechsel, D., Kirschner, M. W. & Martin, D. W. (1989) *Mol. Cell. Biol.* **9**, 1381–1388.
9. Lee, G., Cowan, N. & Kirschner, M. (1988) *Science* **239**, 285–288.
10. Himmler, A. (1989) *Mol. Cell. Biol.* **9**, 1389–1396.
11. Binder, L. I., Frankfurter, A. & Rebhun, L. I. (1985) *J. Cell Biol.* **101**, 1371–1378.
12. Papasozomenos, S. Ch. & Binder, L. I. (1987) *Cell Motil. Cytoskel.* **8**, 210–226.
13. Loomis, P. A., Howard, T. H., Castleberry, R. P. & Binder, L. I. (1989) *J. Cell Biol.* **109**, 77a (abstr.).
14. Brinkley, B. R., Zinkowski, R. P., Mollon, W. L., Davis, F. M., Pisegna, M. A., Pershouse, M. & Rao, F. M. (1988) *Nature (London)* **336**, 251–254.
15. Mitchison, T. J. & Kirschner, M. W. (1985) *J. Cell Biol.* **101**, 755–765.
16. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
18. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4354–4356.
19. Kosik, K. S., Orrechio, L. D., Binder, L. I., Trojanowski, J. Q., Lee, V. M.-Y. & Lee, G. (1988) *Neuron* **1**, 817–825.
20. Galloway, P. G., Perry, G., Kosik, K. S. & Gambetti, P. (1987) *Brain Res.* **403**, 337–340.
21. Kowall, N. W. & Kosik, K. S. (1987) *Ann. Neurol.* **22**, 639–643.
22. De la Torre, C. & Gimenez-Martin, G. (1982) in *The Nucleolus*, eds. Jordan, E. G. & Cullis, C. A. (Cambridge Univ. Press, Cambridge, England), pp. 153–177.
23. Hernandez-Verdun, D., Hubert, J., Bourgeois, C. A. & Bouteille, M. (1978) *C. R. Hebd. Seances Acad. Sci. Ser. B* **287**, 1421–1423.
24. Bourgeois, C. A., Hernandez-Verdun, D., Hubert, J. & Bouteille, M. (1979) *Exp. Cell Res.* **123**, 449–452.
25. Goessens, G. & Lepoint, A. (1982) *Biol. Cell.* **43**, 139–142.
26. Howell, W. M. (1982) in *The Cell Nucleus*, ed. Busch, H. (Academic, New York), Vol. 11, pp. 70–79.
27. Miller, D. A., Dev, V. G., Tantravahi, R. & Miller, J. (1976) *Exp. Cell Res.* **101**, 235–238.
28. Lepoint, A. & Goessens, G. (1978) *Exp. Cell Res.* **117**, 89–94.
29. Ploton, D., Thiry, M., Menager, M., Lepoint, A., Adnet, J.-J. & Goessens, G. (1987) *Chromosoma* **95**, 95–97.
30. Henderson, A. S., Warburton, D. & Atwood, K. C. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3394–3398.
31. Hernandez-Verdun, D. (1983) *Biol. Cell.* **49**, 191–202.
32. Viereck, C., Tucker, R. P., Binder, L. I. & Matus, A. (1988) *Neuroscience* **26**, 893–904.
33. Evans, R. M. & Hollenberg, S. M. (1988) *Cell* **52**, 1–3.
34. Iqbal, K., Zaidi, T., Thompson, C. H., Mertz, P. A. & Wisniewski, H. M. (1984) *Acta Neuropathol.* **62**, 167–177.
35. Kidd, M. (1963) *Nature (London)* **197**, 192–193.
36. Terry, R. D. (1963) *Neuropathol. Exp. Neurol.* **22**, 629–642.
37. Wisniewski, H. M. & Iqbal, K. (1980) *Trends Neurosci.* **3**, 226–228.
38. Wisniewski, K. E., Wisniewski, H. M. & Wen, G. Y. (1985) *Ann. Neurol.* **17**, 278–282.