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Cytogenetics and cell biology of nucleolar tau.

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THE CYTOGENETICS AND CELL BIOLOGY OF NUCLEOLAR TAU

by

VIRGINIA CAROL THURSTON

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION
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Title The Cytogenetics and Cell Biology of Nucleolar Tau

Tau is a neuronal microtubule-associated protein (MAP) which localizes primarily in the axons and somatodendritic compartment of neurons (Binder et al. 1985; Papasozomenos and Binder 1987). In this study we have described our discoveries of the localization of tau outside the central nervous system (CNS), upregulation of tau in actively dividing cells, and confirmation of tau in the nucleolus. Tau proteins appear to have functions in addition to cross-linking and stabilizing microtubules within the axon. Nuclear tau was localized via the Tau-1 monoclonal antibody to nucleoli and nucleolar organizer regions (NORs) of acrocentric chromosomes within HeLa cells and in primary cultures of human fibroblasts, lymphocytes, and lymphoblasts. We also present evidence indicating that Tau-1 staining may be a heritable characteristic reminiscent of silver staining and that Tau-1 immunostaining is upregulated in dividing lymphocytes. Transfection experiments involving tau sense plasmids demonstrated an increase in nucleolar tau in a subset of cells as compared to endogenous tau by quantitative image analysis. Transient transfection of tau anti-sense plasmids abolished nucleolar Tau-1 staining. This further substantiated the presence of tau in the nucleolus of neuronal cell lines, as well as nonneuronal human cells, and strongly suggested that Tau-1 nucleolar staining was due to the presence of tau proteins and not some cross-reacting species.

These studies have contributed to the growing evidence that tau was a multifunctional protein found not only associated with microtubles in the axons and somatodendritic compartments of neurons, but also localized to the nucleolus and NORs in neuroblastomas, HeLa cells, human skin fibroblasts, and lymphocytes.

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NUCLEOLAR LOCALIZATION OF THE MICROTUBULE-ASSOCIATED PROTEIN TAU IN NEUROBLASTOMAS USING SENSE AND ANTI-SENSE TRANSFECTION STRATEGIES

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AMD	actinomycin D
cDNA	complementary deoxyribonucleic acid
CG	human neuroblastoma cells
CHO	Chinese hamster ovary cells
CNS	central nervous system
DS	Down's syndrome
DMEM	Dulbecco's modified Eagle medium
DRB	5,6-dichloro-1-B-D-ribofuranosylbenzimidazole
FITC	fluorescein isothiocyanate
HeLa	human cervical carcinoma cells
IgG	immunoglobulin G
IgM	immunoglobulin M
JC	human neuroblastoma cells
Kb	kilobase
K_d	dissociation constant
kDa	kilodalton
M	molar
MAb	monoclonal antibody

LIST OF ABBREVIATIONS (Continued)

MAP	microtubule-associated protein
mM	millimolar
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
MT	microtubule
NFT	neurofibrillary tangle
NLS	nuclear localization signal
NOR	nucleolar organizer region
µg	microgram
µl	microliter
µm	micrometer
mg	millogram
ml	milliliter
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pH	percentage hydronium ions
PHFs	paired helical filaments
PIPES	piperazine N' N' diethanesulfonic acid
PMSF	phenylmethylsufonylfluoride
PNS	peripheral nervous system
SDS	sodium dodecyl sulfate

LIST OF ABBREVIATIONS (Continued)

TRIS	tris-hydroxymethylaminomethane
UTR	untranslated regions

INTRODUCTION

Microtubule-associated proteins

Microtubules are a major structural element of the cytoplasmic matrix of eucaryotic cells. These hollow, cylindrical fibrils have been found to be composed of protofilaments which consisted of linearly arranged pairs of alternating alpha and beta tubulin subunits (for review see Hirokawa 1991). Microtubules were determined to be intrinsically polar, one end of the microtubule favored for assembly over the other, creating a "plus" and "minus" end, respectively (Bergen and Borisy 1980). Weisenberg (1972) discovered that microtubules could be assembled in vitro from extracts of brain tissue. Examination of the microtubule fraction, enriched by repeated cycles of temperature dependent assembly and disassembly, led to the discovery of a heterogeneous class of proteins known as microtubule-associated proteins (MAPs) (Sloboda et al. 1975). These proteins copurified with tubulin and by electron microscopic analysis were found to decorate the outer wall of the microtubule (Shelanski et al., 1973; Borisy et al. 1975; Sloboda et al. 1976; Murphy et al. 1977). MAPs can be divided into two main groups, the structural MAPs and the motor MAPs. The structural MAPs consist of the high molecular weight MAPs, which include MAP1, MAP2, and MAP4 ($M.W._{App}$ 200-350 kDa), and the low molecular weight MAPs ($M.W._{App}$ of 45-65 kDa), consisting largely of the tau proteins. The motor MAPs consist of two extensive superfamilies of ATPase driven motors, the kinesins and the dyneins.

Analysis of the structural MAPs revealed that these molecules appeared to contain two functional domains, a microtubule binding domain and a projection domain. Two classes of microtubule binding domains have been discovered, a (K/R) (K/R) (E/D) motif repeated 11-21 times within the amino domain of the MAP1 family of proteins (Noble et al. 1989; Langkopf et al. 1992) and a 31-32 amino acid containing motif repeated 3-4 times within the carboxy domains of MAP2, MAP4, and tau proteins (Lewis et al. 1988; Aizawa et al. 1990). Different classes of microtubule binding domains may allow more than one population of MAP to interact noncompetitively with a single microtubule (Shiomura and Hirokawa 1987b; Hirokawa et al. 1988). Electron microscopy of in vitro polymerized microtubules or in vivo microtubules revealed thin filamentous arms extending from the surface of the microtubules, apparently representing the projection domains of the bound MAPs (Murofushi et al. 1986; Shiomura and Hirokawa 1987a; Hirokawa et al. 1988; Sato-Yoshitake et al. 1989; Chen et al. 1992). The projection domain for the MAP1 family was located in the carboxy domain and was approximately 40 nm in length, whereas the projection domains for MAP2, MAP4, and tau were within the amino domains and were 60 nm, 30 nm, and 19 nm in length, respectively. The projection domains were thought to interact with the various cytoskeletal elements and organelles and to determine the intermicrotubule spacing within neurons (Chen et al. 1992).

One of the most important speculated functions of the structural MAPs was the regulation of neuronal development. The maintenance of neuronal plasticity during neurite outgrowth and the cytoskeletal structural stability of the mature neuron was thought to be controlled by the developmentally regulated expression of the various

classes of MAPs. The MAPs found in the developing brain were quite different, in abundance, form, and their ability to promote tubulin polymerization, from those found in adult brain (reviewed by Matus 1988; Tucker 1990). The switch from early to late MAPs in the maturing brain coincided with the stage of development in which neuronal plasticity began to give way to neuronal stability. This implied an important link between the state of neuronal plasticity and MAP isoform expression.

Tau proteins

Tau proteins were first identified as a family of phosphoproteins that associate with microtubules in vivo and enhance microtubule function and stability in vitro (Weingarten et al. 1975; Connolly et al. 1977). Tau was originally localized within axons of the central nervous system (Binder et al. 1985; Kosik and Finch 1987; Brion et al. 1988; Trojanowski et al. 1989). Subsequently, Papasozomenos and Binder (1987) discovered that following treatment of rat brain sections with alkaline phosphatase, tau could be detected, via the Tau-1 monoclonal antibody, on microtubules and ribosomes in the somatodendritic and glial compartments of the central nervous system (CNS). This result indicated that there were posttranslational modifications involved in the localization of tau within the neuron and that tau's function may extend beyond the microtubule.

The tau proteins have been found to be derived from a single gene located on the long arm of chromosome 17 (Neve et al. 1986) which gives rise to 6 isoforms in the CNS. These isoforms were due to alternative splicing and were developmentally regulated. All six isoforms were found in the adult human brain, but only the smallest isoform was present in the fetal brain (Goedert et al. 1993; Bramblett et al. 1993). These isoforms

ranged in predicted molecular weights from 37 to 46 kDa (Goedert et al. 1989) as the result of inclusion or exclusion of four alternatively spliced exons in a 6 kb RNA precursor (Lee et al. 1988; Goedert et al. 1989; Himmler 1989; Himmler et al. 1989). In contrast, tau from the adult peripheral nervous system (PNS) consisted of isoforms with apparent molecular weights ranging from 110-125 kDa (Drubin et al. 1984, 1988; Oblinger et al. 1991).

Tau heterogeneity on SDS PAGE was the result of four alternatively spliced exons in a 2 or 6 kb RNA precursor for CNS tau and an 8 kb RNA precursor for PNS tau (Lee et al. 1988; Goedert et al. 1989; Himmler 1989; Himmler et al. 1989; Couchie et al. 1992; Goedert et al. 1992b). Sadot et al. (1994) discovered two polyadenylation signals in the rat tau 3′-untranslated region. The first signal was located between nucleotides 1359 and 1364 within intron 13/14, which may yield the cDNA sequence described previously for the bovine tau and encodes for a 2 kb species (Himmler et al. 1989). The level of the 2 kb tau mRNA species observed in RNA preparations from human and rat sources was variable and less abundant than the 6 kb species (Goedert et al. 1988, et al. 1992a; Wang et al. 1993). This may result from a lack of stabilizing signals thought to reside in the distal part of the 3′ untranslated region. The second poly-adenylation signal was located between nucleotides 4953 and 4958 and may yield the 6 kb tau transcripts described in the literature (Goedert et al. 1988; Wang et al. 1993).

Each isoform of tau consisted of a variable amino-terminus characterized by the absence or presence of a 29 (exon 2) or 58 (exons 2 and 3) amino acid insert and either three or four (exon 10) highly conserved tandem repeats in the carboxy-terminal half of

the molecule for the CNS tau. These tandem repeats in the carboxy terminal domain are a stretch of 18 amino acids which have been repeated imperfectly three (juvenile tau) or four (adult tau) times and have been demonstrated to be the microtubule binding domain (Himmler et al. 1989; Ennulat et al. 1989; Lee et al. 1989). The PNS tau also contained an N-terminal domain insert of 237-254 amino acids (exon 4a).

As mentioned earlier, the tau proteins were derived from a single gene located on the long arm of chromosome 17 (Neve et al. 1986). This gene was found to be over 100 kb and to consist of 16 exons. Exon -1 was located in the 5' untranslated region. Exons 1, 4, 5, 7, 9, 11, 12, and 13 were constitutively expressed. Exons 2, 3, and 10 were under developmental regulation in most, but not all, regions of the CNS. Exons 4a, 6, and 8 are specifically expressed in certain neuronal subtypes, including the peripheral nervous system (Goedert et al. 1992b; Couchie et al. 1992). Exon 10 is a cassette exon of 31 amino acids that corresponds to one of the repeated microtubule-binding sequences. The inclusion of exon 14 depends on the use of a 5' splice site in exon 13, the regulation of which is unclear (Andreadis et al. 1992).

Although it was apparent that these tandem repeats in the carboxy-terminus of tau were involved in microtubule binding (Drubin and Kirschner 1986; Kanai et al. 1989; Lee et al. 1989), until recently, no specific function for the variable amino-terminal domain had been described. In 1994, Greenwood et al. demonstrated that tau was phosphorylated on Thr^{39} by casein kinase II. They proposed that the phosphorylation site at Thr^{39} in the amino terminus of tau may be a point of regulation for the interaction of microtubules with other cytoskeletal proteins and thus regulate the stability of the cytoskeleton. Recently, Brandt et al. (1995) observed that tau interacted with components at the

cytoplasmic face of the neural plasma membrane through its amino-terminal domain. They hypothesized that an integral or an intracellular membrane-bound protein existed which was bound to tau, enabling tau to bridge microtubules to membranes. Their results suggested that the association of tau's amino-terminal domain with the plasma membrane constituted an important step in neuritic development.

The presence of the extra microtubule-binding domain in adult tau was thought to contribute to its increased microtubule assembly-promoting ability as compared with the juvenile tau (Scott et al. 1992). Juvenile tau was found to be a single species with an apparent molecular weight of 48-52 kDa (Cleveland et al. 1977a; Couchie and Nunez 1985) and was also thought to be axon specific (Brion et al. 1988), but was subsequently immunolocalized throughout the somata, dendrites, and axons (Ferreira et al. 1987; Binder et al. 1985). Juvenile tau was found to have fewer microtubule-binding domains and to exhibit a higher degree of phosphorylation than adult tau. This decreased its ability to promote the assembly and stabilization of microtubules (Lindwall and Cole 1984). Tau promoted GTP-binding to the exchangeable site in the β-subunit of tubulin (Khatoon et al. 1995). Tau specifically increased the frequency of elongation (rescue) and decreased the frequency of rapid shortening (catastrophe) of microtubules by increasing their elongation rate (Drechsel et al. 1992). Purified tau increased the elongation rate of microtubules by decreasing the tubulin subunit dissociation rate (Pryer et al. 1992). The tandem repeats in the carboxy region of tau were involved in microtubule binding at a level of one tau molecule/two tubulin dimers (Gustke et al. 1994).

More recently, a high-affinity microtubule-binding site in the region between the first and second repeats (which is only found in the 4 repeat form) has been identified. This inter-repeat region associated directly with tubulin at sites distinct from the repeated binding domain (Goode and Feinstein 1994). Gustke et al. (1994) suggested that regions on either side of the repeat domains, the proline-rich domain on the amino side and on the carboxy side, a region loosely homologous to the repeats, contributed substantially to the binding of microtubules, forming "jaws" that capture the MAP-binding domain of tubulin. Tau constructs containing repeat domains alone bind poorly, while constructs that lack repeats bind quite well, although this tau is a weak nucleator of microtubule assembly. Other constructs containing the parts to the left and right of the repeats show negligible binding by themselves, indicating the need for a large combination of the repeat domains and the regions flanking them for tight binding.

Tau was found to be an elongated molecule with a length around 35 nm, but without a well defined shape (Willie et al. 1992). It has been described as a protomer with a random coil structure (Schweers et al. 1994), although recently, Carmel et al. (1996) demonstrated a model of noncontinuous antibody selectivity in predicting that the N-terminus of tau is in close association with its microtubule repeat region. Presumably, monomeric tau can adopt this conformation due to its flexibility, but the energy required to do so is reflected in a higher K_d value. This conformation is detected using the antibody Alz50, which labels distinct populations of neurons in normal human brain, fetal brain, and early stage neurofibrillary degeneration, indicating that Alz50 selectivity recognizes a distinct subset of tau proteins presumably in the polymeric or prepolymeric state. Native tau was found to be phosphorylated at about 2 phosphates/mole, which was

distributed over several sites (Ksiezak-Reding et al. 1992; Watanabe et al. 1993; Kopke et al. 1993). It was thought that this phosphorylation somehow regulated tau's affinity for microtubules in that hyperphosphorylated tau did not bind microtubules as well as normal tau (Drechsel et al. 1992).

Structure of the tau gene

Until recently the 5' untranslated region and the promoter region of the tau gene had not been cloned. Andreadis et al. (1996) discovered an abundance of C and G residues and the absence of TATA and CAAT boxes in the region upstream of the human tau gene; in comparison with the 5' UTR of the rat gene (Sadot et al. 1994), the human tau gene showed 91% identity. These features have been normally encountered in housekeeping genes (Gardiner-Garden and Frommer 1987). The promoter region contained binding sites for two general (SP1 and GCF) and one specific transcription (AP2) factor. SP1 was a general activator (Su et al. 1991), GCF was a repressor of GC-rich promoters (Kageyama and Pastan 1989), and AP2 was a transcriptional activator most abundant in cells of the neural crest lineage (Falconer et al. 1992). Reporter gene results, as well as RT-PCR and primer extensions, suggested that the tau promoter directly upstream from exon -1 was not neuron specific.

Sadot et al. (1996) isolated and characterized the 5'-flanking region of the tau gene from a rat P1 library (Sternberg 1990). They also discovered that the putative tau promoter region was a TATA-less, G + C-rich promoter that directed multiple initiation sites of transcription. Three SP1-binding sites were present in the proximity of the first transcription initiation site and have been shown to be important in directing transcription initiation in other TATA-less promoters (Lu et al. 1994). RNase-protection assays were

performed to determine the main transcription initiation sites. In exon -1, three initiation sites were located at positions 1, 25, and 69 (as numbered by Sadot et al. 1996). Truncated fragments from the promoter region were ligated upstream of the luciferase reporter gene (Deng and Karin 1993) and their promoter activity was tested. No tau promoter activity was found in transfected nonneuronal COS cells, but in transfected PC12 cells, promoter activity was increased 8.5- to 12-fold, indicating that tau promoter activity was positively regulated in neuronal cells. The promoter may be composed of two different elements as illustrated by the stepwise decrease in luciferase activity seen in the three short truncations.

Nonmicrotubule associations of tau

Tau proteins appear to have functions in addition to cross-linking and stabilizing microtubules within the axon. The outer membrane of rat brain mitochondria appeared to have specific binding sites for tau, although the orientation of the tau molecule on the mitochondria is still controversial (Jancsik et al. 1989; Leterrier et al. 1990; Rendon et al. 1990; Jung et al. 1993). Ribosomes can also bind tau (Papasozomenos and Binder 1987). Recently, tau has been found to be involved in the activation of phospholipase C (PLC), which in turn hydrolyzed phosphatidylinositol 4,5-bisphophate (Hwang et al. 1996). This reaction is one of the earliest key events in the regulation of cellular function. It now appears that tau proteins are multifunctional, involved not only with stabilizing the cytoskeleton, but also in organizing various intracellular components.

Tau in Alzheimer's disease

Tau has also been found in the neurofibrillary tangles (NFTs) characteristic of Alzheimer's Disease (AD). The filamentous lesions (NFTs) that have been diagnostic of

AD occur within the neurons, in extracellular deposits (neuritic plaques), and in meningeal and cerebral blood vessels (amyloid angiopathy) (reviewed by Selkoe 1991). The NFTs were found to be of particular importance because their density in brain is closely correlated with the severity of dementia (Crystal et al. 1988; Dickson et al. 1988; Barcikowska et al. 1989; Dickson and Yen 1989). Immunochemical and biochemical analysis of AD brains revealed that the major components of the NFTs were paired helical filaments (PHFs) (Kidd 1963, 1964) which were composed of polypeptides with apparent molecular weights of 60, 64, and 68 kDa (Grundke-Iqbal et al. 1986; Kosik et al. 1986; Greenberg and Davies 1990). These polypeptides have subsequently been identified as abnormally phosphorylated isoforms of tau (Grundke-Iqbal et al. 1986; Greenberg and Davies 1990; Lee et al. 1991; Hasagewa et al. 1992; Goedert et al. 1993). All the major sites of abnormal phosphorylation appeared to be Ser/Pro or Thr/Pro sites (Lang et al. 1992; Otvos et al. 1994; Szendrei et al. 1993; Goedert et al. 1994; Hasagewa et al. 1992).

In 1992, additional studies showed that the phosphorylation state of PHF tau partially recapitulated the phosphorylation state of fetal human and rat CNS tau (Kanemaru et al. 1992; Yoshida and Ihara 1993). For example, Ser^{202} and Ser^{396} were shown to be phosphorylated in PHF tau and in the smallest tau isoform isolated from postmortem samples of the fetal human CNS, but not in any of the six tau isoforms isolated from postmortem samples of the normal adult CNS (Bramblett et al. 1993). Now, important new data have suggested that freshly isolated adult human biopsy CNS tau were phosphorylated at almost all of the same sites as PHF tau, although to a lesser extent than PHF tau (Matsuo et al. 1994; Mawal-Devan et al. 1994). Still, excessively

phosphorylated PHF tau obtained from autopsy brain appeared to be unable to bind to microtubules (Bramblett et al. 1993; Yoshida and Ihara 1993); therefore, it was plausible to infer that the conversion of normal tau into PHF tau could alter the microtubule-binding ability of tau, thus destabilizing microtubules, which would lead to disruption of axonal transport and ultimately to neuronal death in AD (Trojanowski and Lee 1994).

Nuclear tau

Nonmicrotubule localization of tau has also been reported by Loomis et al. (1990). Human neuroblastoma cells analyzed with the Tau-1 monoclonal antibody (Binder et al. 1985) displayed anti-tau immunoreactivity in the fibrillary regions of interphase nucleoli and on the nucleolar organizer regions (NORs) of the acrocentric chromosomes (13, 14, 15, 21, and 22). The nucleolus was found to be an intranuclear organelle where ribosomal rRNA was transcribed and ribosome assembly began (reviewed by Busch and Smetana 1970; Goessens 1984; Hadjiolov 1985; Hernandez-Verdun 1991). During mitosis, Tau-1 localized to the distal regions of the short arms of the acrocentric chromosomes, the site of the rDNA genes (reviewed in Goessens 1984). These chromosomal locations were also referred to as the nucleolar organizer regions (NORs). The localization patterns of tau suggested it may play a role in structuring the interphase nucleolus and reformation of the nucleolus after cell division. Due to the fact that the Tau-1 monoclonal antibody was the only tau antibody to date to react with nucleoli and NORs by immunofluorescence, there was still some controversy over tau's presence in this organelle.

The presence of tau in the nucleus was recently reported in nuclei isolated from frozen human brain using three monoclonal antibodies (Brady et al. 1995). The staining

of the brain nuclei was largely punctate and extranucleolar, although some nucleolar staining was occasionally observed with all three antibodies employed. Differences in immunolocalization patterns may reflect differences in cell cycle stage and/or cell differentiation. The lack of nucleolar tau in certain terminally differentiated cells such as neurons suggested that its functions were no longer necessary and that the small number of nuclei displaying nucleolar tau may be undergoing intense changes in rRNA synthesis (Brady et al. 1995).

Nuclear tau, similar to certain populations of PHF tau, resists solubilization by conventional methods and must first be extracted with formic acid prior to solubilization in SDS-electrophoresis sample buffer. Furthermore, primate cells expressing nuclear tau contained a novel 2 kb tau message in addition to low levels of the well characterized 6 kb isoform (Wang et al. 1993). The 2 kb message was not derived from further processing of the 6 kb but rather from the original tau mRNA transcript. The 2 kb message had a coding region identical to that of the 6 kb tau message, indicating that the untranslated regions of the two mRNAs account for the variability in message size.

There had remained some controversy over the presence of tau in the nucleus, although some transfection work had been done with tau sense and tau anti-sense oligonucleotides in rat neuroblastoma cells (Lambert et al. 1995). These studies used the monoclonal antibodies PHF-1 and Alz-50 to determine the presence of tau in transfected cells. The PHF-1 epitope was found in the nucleus and the cytoplasm, while the Alz-50 epitope was excluded from the nucleus of these cells. Compared to cells given tau sense oligonucleotides, cells given tau anti-sense had dramatically decreased PHF-1 staining. Nuclear as well as cytoplasmic staining was eliminated (Lambert et al. 1995). No

indication was given by the authors of what effect tau sense transfections had on nuclear tau.

Additional characterization of the ability of tau to associate with nuclear components was carried out by Lu and Wood (1993). When fluorescently labeled bovine tau was microinjected into Chinese hamster ovary cells (CHO), which contained no tau mRNA or tau protein (Wang et al. 1993), the tagged protein localized to both the centrosome and the nucleolus. Unlike centrosomal staining, which decreased over time, the strong nucleolar staining by tau was maintained throughout the entire experimental time period, indicating that it was targeted directly to the nucleolus, where it functioned in a different capacity than cytoplasmic tau.

Greenwood and Johnson (1995) also analyzed tau immunoreactivity in LA-N-5 neuroblastoma cells and confirmed by indirect immunofluorescence the presence of tau in the nucleolus. Extraction of tau from the LA-N-5 nuclei in the chromatin fraction suggested the association of nuclear tau with DNA, either directly or indirectly. This extraction procedure allowed an examination of the phosphorylation state of nuclear tau compared with cytosolic tau, which demonstrated that nuclear tau was phosphorylated to the same extent as cytosolic tau. This method also suggested that the actual phosphorylation event occurred in the cytosol prior to transport into the nucleus and that nuclear tau comprised 16% of total tau protein in the LA-N-5 cells (Greenwood and Johnson 1995).

The objective of this research project was to further document the location and the presence of tau in the nucleolus. Although tau's presence in the nucleus was well established, with rare exception, only the Tau-1 antibody stains the nucleolus.

Surprisingly, this is a neglected area in the fields of both microtubule-associated and nuclear proteins. This is likely due to our inability to assign a function for a well established cytoplasmic protein in the nucleus and nucleolus. Due to the problems in isolating nuclear tau, little is known of its functional role in the nucleus. Sections of this dissertation report non-CNS and nonmicrotubule localizations of tau. Tau was localized via the Tau-1 monoclonal antibody to nucleoli and NORs of acrocentric chromosomes in cells outside the central nervous system, including HeLa cells, and in primary cultures of human fibroblasts, lymphocytes, and lymphoblasts. Its immunofluorescence intensity varied between NORs of the acrocentric chromosomes but remained constant on specific chromosomes in cells obtained from the same individual. This pattern was reminiscent of, although not identical to, silver-staining patterns that indicated transcriptionally active rRNA genes in the previous interphase (Goodpasture and Bloom 1975; Howell et al. 1975). We have also presented evidence indicating that Tau-1 staining may be a heritable characteristic, also similar to silver staining, and that Tau-1 immunostaining was upregulated in dividing lymphocytes.

The last section of this dissertation describes additional transfection studies using tau sense and tau anti-sense expression plasmids. We used indirect immunofluorescence to analyze human neuroblastoma cells that contained nucleolar tau (CG cells). Transient transfection of tau sense plasmids induced the expression of cytoplasmic tau as well as an increase in nucleolar tau in a subset of cells as compared to endogenous tau as confirmed by quantitative image analysis. Transient transfection of tau anti-sense plasmids abolished nucleolar Tau-1 staining in these cells. This work further substantiated the presence of tau in the nucleolus of neuronal cell lines as well as nonneuronal human cells,

and strongly suggested that Tau-1 nucleolar staining was due to the presence of tau proteins and not some cross-reacting species. Furthermore, Tau-1 immunoreactivity may provide a novel, silver-stain-like cytogenetic marker.

TAU AS A NUCLEOLAR PROTIEN IN HUMAN NONNEURAL CELLS IN VITRO AND IN VIVO

by

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ABSTRACT

The Tau-1 monoclonal antibody was localized to the nucleolus of interphase cells and the nucleolar organizing regions (NORs) of acrocentric chromosomes in cultured human cells. Putative nucleolar and NOR tau was found in HeLa cells and lymphoblasts as well as in nontransformed fibroblasts and lymphocytes. To confirm the presence of tau in the nuclei of these nonneural cells, immunoblot analysis was performed on isolated nuclei from lymphoblasts. Several tau bands were noted on the blot of the nuclear extract suggesting the presence of multiple tau isoforms. Tau-1 immunostaining demonstrated variable staining intensities between individual acrocentric chromosomes in all cells tested. In cultured peripheral lymphocytes, these staining patterns were the same from one chromosome spread to the next within an individual. This consistency of Tau-1 staining and its variability among NORs was reminiscent of staining patterns obtained using the silver-NOR procedure. Comparisons of Tau-1 immunostaining with silver staining of chromosome spreads from human lymphocytes demonstrated that Tau-1 did not immunostain all of the NORs that were silver stained. The intensity of Tau-1 fluorescence in nucleoli was further shown to be increased in phytohemagglutinin-stimulated lymphocytes, indicating an upregulation of nuclear tau when cells reentered the cell cycle. These results contribute to a growing body of evidence defining tau as a multifunctional protein that may be involved in ribosomal biogenesis and/or rRNA transcription in the nucleus of all cells as well as microtubule-stabilizing functions in the neuronal cytoplasm.

INTRODUCTION

Tau is predominantly a neuronal microtubule-associated protein (MAP) that is involved in microtubule stability (Binder et al. 1985; Drubin and Kirschner 1986). It is mainly found in the axons and somatodendritic compartment of neurons (Binder et al. 1985; Papasozomenos and Binder 1987) although glial localization has also been reported (Papasozomenos and Binder 1987; Migheli et al. 1988; LoPresti et al. 1995). Structurally, tau is a tripartite phosphoprotein composed of a variable N-terminal domain, a constant central domain, and a microtubule-binding domain in the C-terminal region. In the adult human brain six isoforms are found, which range from 352 to 441 amino acids and result from alternate splicing of a single mRNA transcript (Lee et al. 1988; Himmler 1989; Himmler et al. 1989). As such, tau isoforms can contain either three or four microtubule-binding domains as well as a 29 or 58 amino acid insert in the amino-terminal region (Goedert et al. 1989; Himmler 1989; Himmler et al. 1989). Tau has been found to be a flexible molecule owing in part to its pro-gly motifs (Lichtenberg et al. 1988). This flexibility results in the presentation of different epitopes responsible for differential staining patterns with various monoclonal tau antibodies (Loomis et al. 1990; Brady et al. 1995; LoPresti et al. 1995).

Although tau was first described as a MAP, this protein has also been observed in other locations in recent years. Hyperphosphorylated tau is the major component of paired helical filaments (PHFs), which represent abnormal structures found in the hippocampus and select cortical regions of individuals suffering from Alzheimer's Disease (AD) (Grundke-Iqbal et al. 1986; Ksiezak-Reding et al. 1990; Greenberg et al. 1992). PHF tau often contains four times as much phosphate as normal tau, and is

extremely insoluble even in SDS-containing buffers (Ihara et al. 1983; Iqbal et al. 1984; Wischik et al. 1988). Immunocytochemistry experiments have demonstrated tau on ribosomes in the somatodendritic compartments of neurons and glial cells (Papasozomenos and Binder 1987), and abnormally phosphorylated tau has been identified on ribosomes of neurons and astrocytes in AD brain (Papasozomenos 1989, 1991). Previously, our laboratory has demonstrated that the tau monoclonal antibody, Tau-1, stained the fibrillar regions of interphase nucleoli and the nucleolar organizer regions (NORs) of acrocentric chromosomes of dividing primate cells in culture (Loomis et al. 1990; Wang et al. 1993). Nucleoli are the sites of ribosome biogenesis while the NORs on the short arms of human acrocentric chromosomes (nos. 13, 14, 15, 21, and 22) represent the chromosomal sites of rRNA genes. The Tau-1 monoclonal antibody (Loomis et al. 1990) recognizes an epitope at amino acids 189-207 on the longest human tau isoform (Kosik et al. 1988). When this epitope is phosphorylated at Ser^{202}, as it is in AD, Tau-1 binding is occluded (Liu et al. 1993). Moreover, our laboratory has demonstrated the presence of tau in nuclei isolated from fresh frozen human brain (Brady et al. 1995). Interestingly, nuclear tau has solubility properties similar to PHF tau, which has been found in both perinuclear and intranuclear locations (Metuzals et al. 1988). Nuclear tau is insoluble in SDS sample buffer, requiring formic acid extraction for solubilization. However, unlike PHF tau, nuclear tau reacted robustly with Tau-1, indicating that it was not phosphorylated at Ser^{202}.

We report here Tau-1 localization in nucleoli within HeLa cells and in primary cultures of human fibroblasts, lymphocytes, and lymphoblasts. That the Tau-1 localization is likely due to tau and not some other cross-reacting protein species was

suggested by immunoblot analysis of lymphoblast nuclei. Both the Tau-1 and Tau-5 antibodies reacted with polypeptides migrating in the tau region of the gel. Tau-1 staining was localized to the NORs and also the fibrillar component of nucleoli in interphase cells. Its immunofluorescence intensity varied between NORs of the acrocentric chromosomes but remained constant on specific chromosomes in cells obtained from the same individual. This pattern was reminiscent of, although not identical to, silver-staining patterns that indicate NOR activity in the previous interphase (Goodpasture and Bloom 1975; Howell 1975). Hence, tau immunostaining may represent a convenient and reliable method to identify some other functional property of NORs.

MATERIALS AND METHODS

Cell Culture

The nontransformed cell line GM03440B (human skin fibroblast, Coriell, Camden, NJ) was cultured in modified Eagle's medium (BioWhittaker, Walkersville, MD) supplemented with 20% fetal bovine serum, 20 mM L-glutamine, 0.1 μg/ml penicillin/streptomycin and 10 mM sodium pyruvate (BioWhittaker). HeLa (human cervical carcinoma) cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% fetal bovine serum. Fresh human lymphocytes were isolated from heparinized whole blood by Ficoll separation and cultured in RPMI 1640 (BioWhittaker) supplemented with 20% fetal bovine serum. For chromosome isolation and phytohemaglutinin (PHA) stimulation experiments, cultures were supplemented with 0.2 μg/ml PHA (GIBCO, Gaithersburg, MD) for 72 h. All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Immunofluorescence

To obtain mitotic cells, Colcemid (0.1 μg/ml) was added to the cultures 3-4 h before harvesting. The cells were collected by centrifugation (200 *g*) and swollen in hypotonic solution (0.075M KCl, 1 mM spermidine HCl, 0.5 mM spermine) for 5 min at 37°C. Cells and isolated chromosomes were then cytocentrifuged onto glass microscope slides using a Shandon Cytospin III (Shadon Southern Instruments, Sewickley, PA) at 500 rpm for 2 min. Cells were subsequently fixed in 3% ultrapure TEM grade formaldehyde (Tousimis Research Corp., Rockville, MD) in 100 mM Tris-HCl, pH 7.4 (Buffer A) for 30 min at 24°C while chromosome spreads were fixed in 100% methanol for 7 min at -20°C. Cells fixed in 3% formaldehyde were permeabilized in 0.5% Triton-X-100 in Buffer A for 5 min and then reduced in 0.5 μg/ml $NaBH_4$ in Buffer A for 4 min. Both cells and chromosomes were then processed for indirect immunofluorescence microscopy (Brinkley et al. 1988). The preparations of cells and chromosomes were counterstained with 1 μg/ml of the DNA stain Hoechst 33258 (Sigma, St. Louis, MO), rinsed in Buffer A and coverslipped with 0.1% *p*-phenylenediamine (Sigma) in a 9:1 mixture of PBS:glycerol.

Quantitation of Immunofluorescence

After the slides were processed for immunofluorescence, video images of mitotic spreads or nucleoli were captured and stored on the computer. For mitotic cells, stage coordinates were recorded so the identical cells could be relocated after fluorescence in situ hybridization (FISH). Samples processed for immunofluorescence were analyzed using a Jenalumar epifluorescence microscope equipped with differential interference contrast (DIC) optics and an Optronics Tec-470 CCD video camera (Goleta, CA) coupled

to an Image-1 (Universal Imaging) image analysis system and an IBM compatible PC 486/33. Image processing was performed by setting the shutter speed and contrast controls on the camera as well as the gain and black level on the imaging program to provide an optimal signal. Then, 128 video images were summed into the foreground frame buffer, and a previously obtained background image was subtracted from the foreground buffer to control for uneven illumination. Image quantification was performed by thresholding the area to be quantitated and subsequently measuring the gray level of each pixel. Each pixel has a gray scale that ranges from 0 to 255. The pixels of each thresholded area were summed and the final measurement was recorded to the hard drive. The resultant data were imported into Excel and intensity of fluorescence of each NOR and nucleolus was analyzed.

Fluorescence In Situ Hybridization

Coverslips were removed from slides containing chromosomes processed for immunofluorescence. The chromosomes were postfixed in 3:1 methanol:acetic acid for 1 h at room temperature. The slides were air-dried overnight, and the following morning processed for FISH using chromosome-specific probes and reagents obtained from Oncor (Gaithersburg, MD). Chromosome spreads were processed according to Oncor's Dual Color Detection in Situ Hybridization system. The slides were stained with 18 μl of 4',6-diamidino-2-phenylindole/Antifade (Oncor), coverslipped, and sealed with nail polish. A biotin-labeled probe (D13Z1/D21Z1) was used to detect the centromeric DNA of chromosomes 13 and 21. This allowed identification of the acrocentric chromosomes 13 and 21 because they were labeled, and chromosome 22 because it is the smallest unlabeled acrocentric. The same metaphase spreads visualized by immunofluorescence

were relocated and individual acrocentric chromosomes were identified. After chromosome identification, the intensity of NOR fluorescence of each chromosome was quantitated on the computer as was the intensity of fluorescent interphase nucleoli. Representative images were printed using a Tektronics Phaser SDXII digital color printer (Beaverton, OR). We were unable to distinguish between chromosomes 14 and 15 because, at the time these experiments were performed, probes specific for these chromosomes were unavailable. Hence the data obtained from chromosomes 14 and 15 were pooled.

Isolation and Extraction of Nuclei from Cultured Cells

Nuclei were isolated from human lymphoblasts using the method of Mitchison and Kirschner (Mitchison and Kirschner 1985). Cells were removed from culture flasks with a cell scraper, rinsed in PBS (150 mM NaCl, 28.7 mM Na_2HPO_4, and 12.8 mM NaH_2PO_4, pH 7.4) and then pelleted by centrifugation (60-100 *g*). The pellet was resuspended in 10 vol. of swelling buffer (5 mM NaCl, 5 mM $MgCl_2$, 5 mM PIPES, 0.5 mM EDTA, pH 7.0) and incubated on ice for 20 min. The cells were pelleted by centrifugation at 2000 *g* for 2 min, resuspended in 5 ml of lysis buffer (10 mM PIPES, 2 mM EDTA, 1% digitonin, 0.1% 2-mercaptoethanol, 1 mM spermidine HCl, 0.5 mM spermine, pH 6.8) and then homogenized with 15 strokes in a Dounce homogenizer using pestle B. The sample was subsequently layered over 15 ml of 40% buffered sucrose (40% sucrose, 10 mM PIPES, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.5 mM spermidine HCl, 0.25 mM spermine, pH 6.8) and pelleted at 2500 *g* for 25 min. The pellet containing isolated nuclei was resuspended in 50 mM TRIS, pH 6.8 and washed three times in TRIS buffer.

Gel Electrophoresis

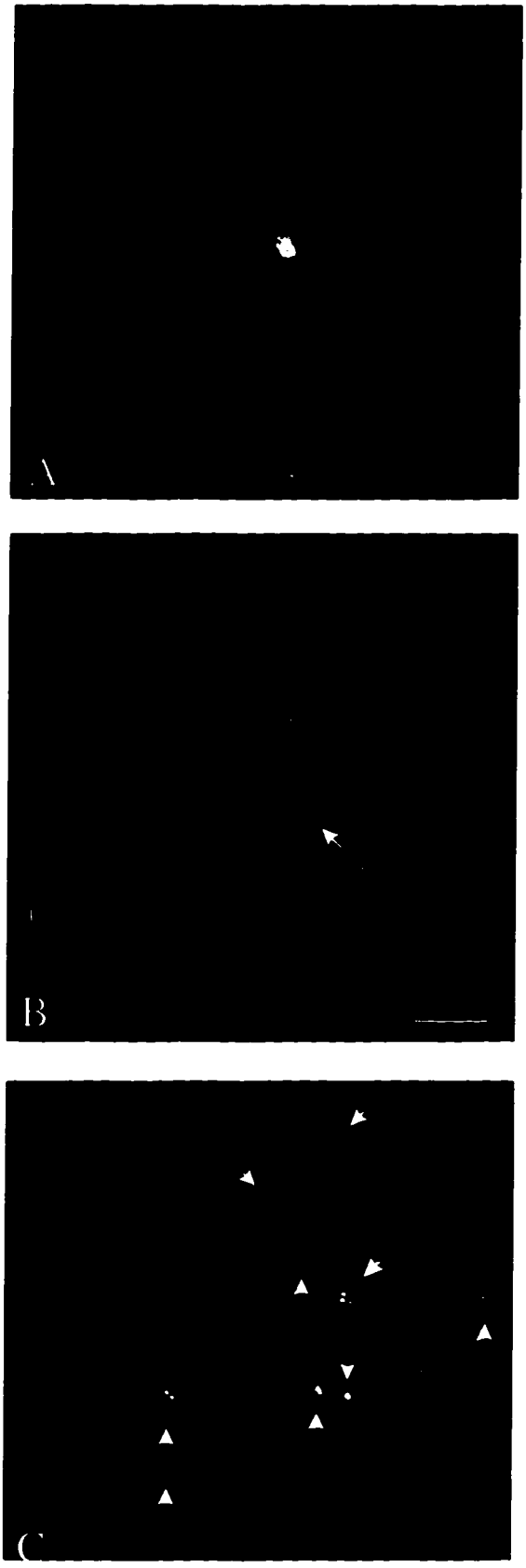
Isolated nuclei were resuspended in 0.1 M TRIS, pH 6.8, extracted for 30 s by the addition of formic acid to a final concentration of 3% and subsequently sonicated for 30 sec. The samples were dialyzed overnight in 0.1 M TRIS, pH 6.8 and then concentrated using an Amicon Centricon-10 concentrator (Amicon Corp., Arlington Heights, IL). The samples were further solubilized by boiling in electrophoresis buffer (62.5 mM TRIS, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) prior to electrophoresis on an SDS 6%-12% polyacrylamide gradient gel as described by Laemmli (1970). The separated proteins were then transferred to nitrocellulose by the method of Towbin et al. (1979) and probed with the tau monoclonal antibodies Tau-1 (Binder et al. 1985) and Tau-5 (LoPresti et al. 1995). Bound antibodies were detected using chemiluminescence (Amersham, Arlington Heights, IL). Protein concentrations were determined by the Bio-Rad Protein Assay (Hercules, Calif.), a modification of the assay of Bradford (1976).

RESULTS

Detection of Nucleolar and NOR Tau-1 Staining in HeLa cells

Nuclear tau has been documented in neuroblastoma cell lines, including the CG neuroblastoma cell line, which has only nuclear tau and entirely lacks cytoplasmic tau (Loomis et al., 1990; Wang et al., 1993). To determine whether nonneural cell lines of human origin also contained nuclear tau and Tau-1 staining was present on NORs during cell division, we conducted further studies using HeLa cells. In interphase HeLa cells, Tau-1 staining appeared to be located in the dense fibrillar regions of the nucleolus (Fig. 1A, B) and on the short arms of acrocentric chromosomes (Fig. 1C, arrowheads). Immunofluorescence demonstrated a variable staining pattern between the NORs of the

Fig. 1. HeLa cells immunostained with Tau-1. **A** HeLa cell at interphase showing Tau-1 staining in the nucleolus and **B** Hoechst staining of the same cell as in **A**. *Arrow* identifies location of Tau-1 immunostain in counterstained nucleus. **C** HeLa chromosome spreads immunostained with Tau-1 and counterstained with Hoechst 33258. *Arrowheads* depict Tau-1 reaction at the nucleolar organizer regions (NORs) of acrocentric chromosomes. Bar represents 10 μm

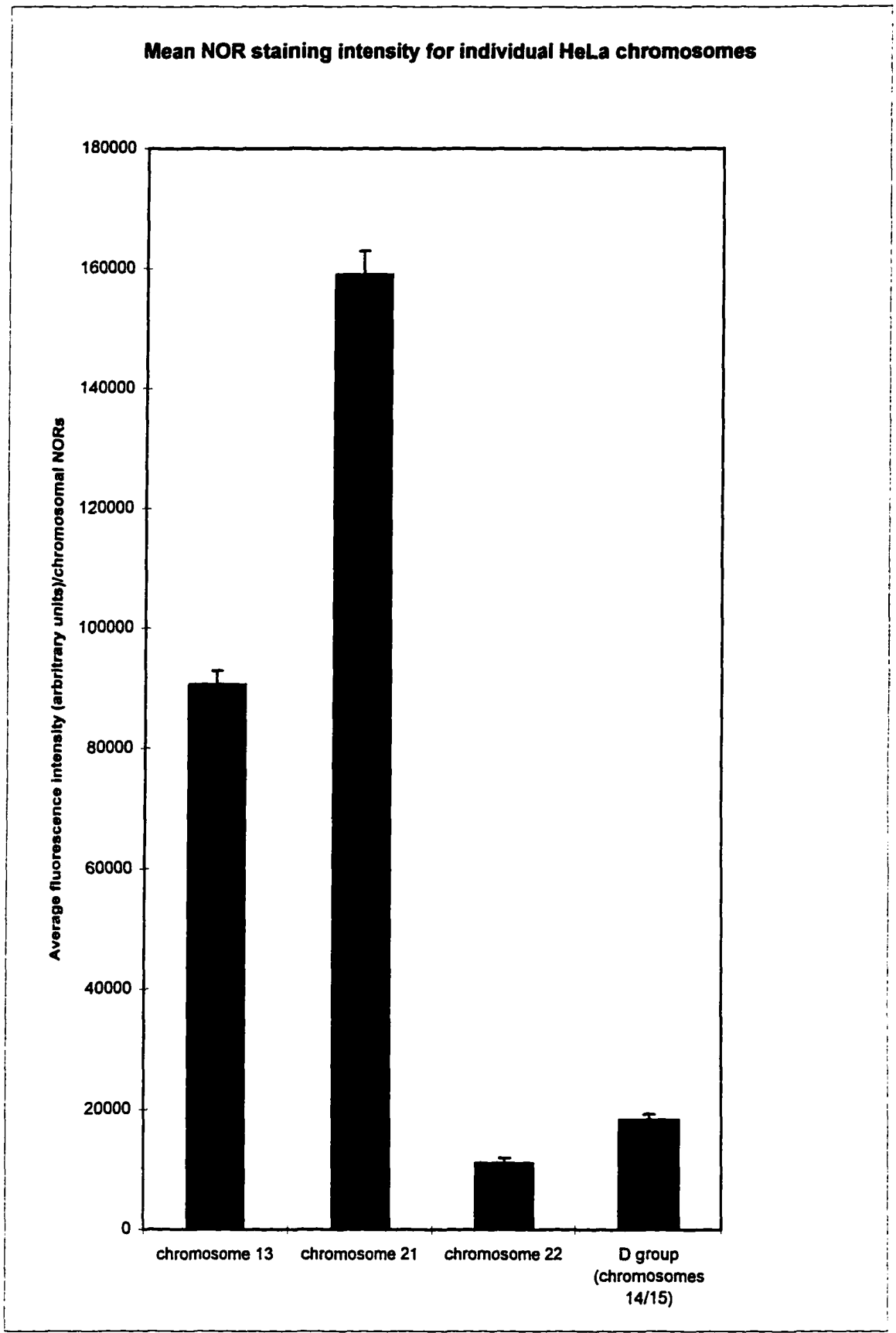


acrocentric chromosomes (Fig. 1C, arrowheads). Therefore, the intensity of fluorescent staining of each individual NOR was quantitated by image analysis. As described in Materials and methods, chromosome spreads were processed for immunofluorescence using Tau-1 and their images and locations recorded on the computer. Next, the chromosome spreads were processed for FISH using a cDNA probe to the centromeric alphoid DNA-containing regions of chromosomes 13 and 21. This allowed ready identification of chromosomes 13 and 21 but also allowed identification of chromosome 22 as the smallest unlabeled autosome. Chromosomes 14 and 15 were pooled as unlabeled D group chromosomes, since no marker probe existed for these chromosomes when the experiments were performed. This combination of Tau-1 staining followed by chromosome identification using FISH allowed measurement of Tau-1 fluorescent intensities per individual chromosome or chromosome group (Fig. 2). The mean intensity of Tau-1 immunostaining varied among NORs of different acrocentric chromosomes in HeLa cells (Fig. 1C). The use of immunofluorescence and FISH methods demonstrated that, in the HeLa cell clone assayed, chromosome 21 contained significantly more tau protein immunoreactivity than any of the other acrocentrics, chromosome 13 had the next highest immunoreactivity, followed by the D group chromosomes and finally chromosome 22 (Fig. 2). These results not only demonstrated the presence of Tau-1 staining on NORs of chromosomes from transformed nonneural human cells, but also demonstrated that staining intensity varied among the different acrocentric chromosomes.

Detection of Nucleolar Tau-1 Staining in Nontransformed Human Fibroblasts

To determine whether nuclear tau was also present in other nonneuronal human cell lines, and whether this was a characteristic of transformation, nontransformed human

Fig. 2. Mean intensity of Tau-1 immunostained NORs of HeLa chromosomes. Owing to the size difference of the acrocentric chromosomes, chromosomes 13, 21, and 22 were identified using fluorescence in situ hybridization (FISH) and a 13/21 centromeric probe. Chromosomes 14 and 15 were indistinguishable, and hence were pooled as group D. The antibody staining intensity of each acrocentric chromosome was measured using quantitative image analysis. Chromosomes 21 and 13 showed a greater intensity of Tau-1 fluorescence than did chromosomes 22 and the D group chromosomes. *Error bars* indicate 1 SD (n=50)



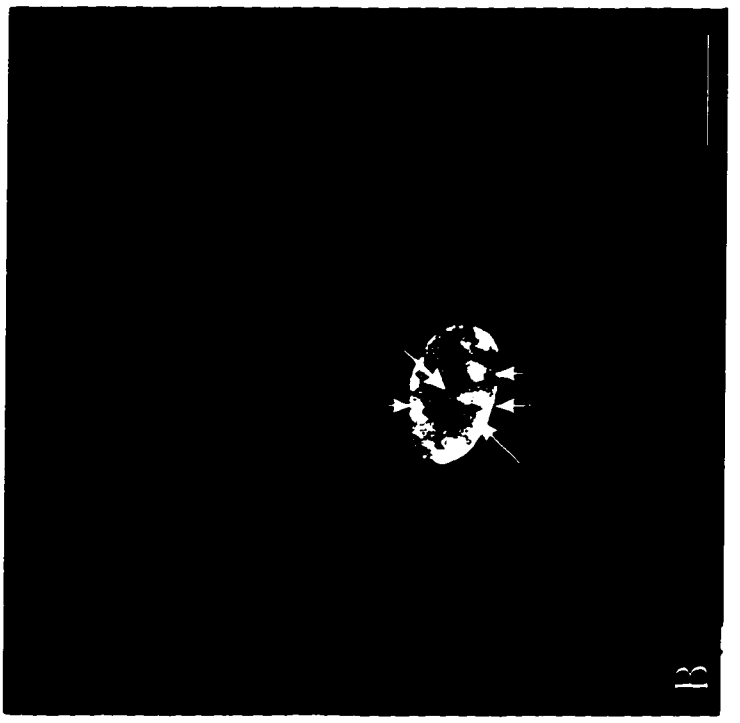
skin fibroblast cell lines were processed for immunofluorescence. Tau-1 stained the nucleolus in interphase fibroblasts, and during mitosis, localized to the short arms of the acrocentric chromosomes. Tau-1 staining in the nucleoli of human fibroblasts was punctate and indicative of localization to the fibrillar regions (Fig. 3A, B). Tau-1 immunostaining was also observed on the NORs of the metaphase chromosomes of human fibroblasts (data not shown), as was the case with cultured neural cell lines (Loomis et al. 1990; Wang et al. 1993). Interestingly, Tau-1 staining varied in intensity from NOR to NOR as discussed above for HeLa cells, and was often absent from some of the acrocentric chromosomes while intensely labeling others (data not shown).

Detection of Nucleolar Tau-1 Staining in Human Lymphocytes

Subsequent experiments were performed to determine whether nuclear tau is present in lymphocytes, since these cells can be readily obtained and are commonly used in human cytogenetic analysis. As was observed in HeLa cells and fibroblasts, Tau-1 staining was also present in interphase nucleoli of human lymphocytes (Fig. 4A-C). Tau staining on the NORs during mitosis (Fig. 4D) varied in intensity from NOR to NOR and was often absent from some of the acrocentric chromosomes (Fig. 4D) while intensely labeling others.

To determine whether Tau-1 staining was consistent from spread to spread in the same individual, chromosome spreads obtained from blood drawn on different days from the same person were analyzed by Tau-1 immunostaining (Fig. 5A, C). Images of individual chromosome spreads were then saved and their location recorded. Subsequently, the same cells were processed for FISH using a 13/21 cDNA probe to the centromeric alphoid DNA regions of chromosomes 13 and 21 (Fig. 5B, D), the same

Fig. 3A, B. Nontransformed human skin fibroblasts immunostained with Tau-1. **A** Fibroblast immunostained with Tau-1. **B** Corresponding Hoechst image of cell in **A**. *Arrows* in **B** point to nucleoli that are labeled with tau in **A**. Bar represents 10 μm



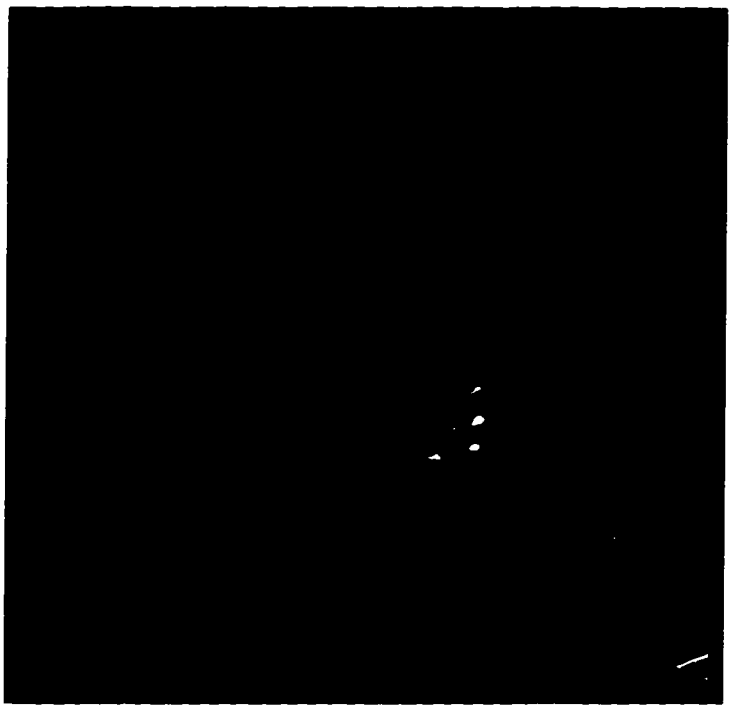


Fig. 4. Human lymphocytes immunostained with Tau-1. **A** Lymphocytes immunostained with Tau-1. **B** Corresponding Hoechst image of **A**. *Arrows* point to nucleoli stained by Tau-1. **C** Corresponding differential interference contrast (DIC) image of **A**. **D** Chromosome spread immunostained with Tau-1. *Arrowheads* point to Tau-1 staining on the NORs during mitosis; *arrows* point to unstained acrocentric chromosomes. Bar represents 25 μm

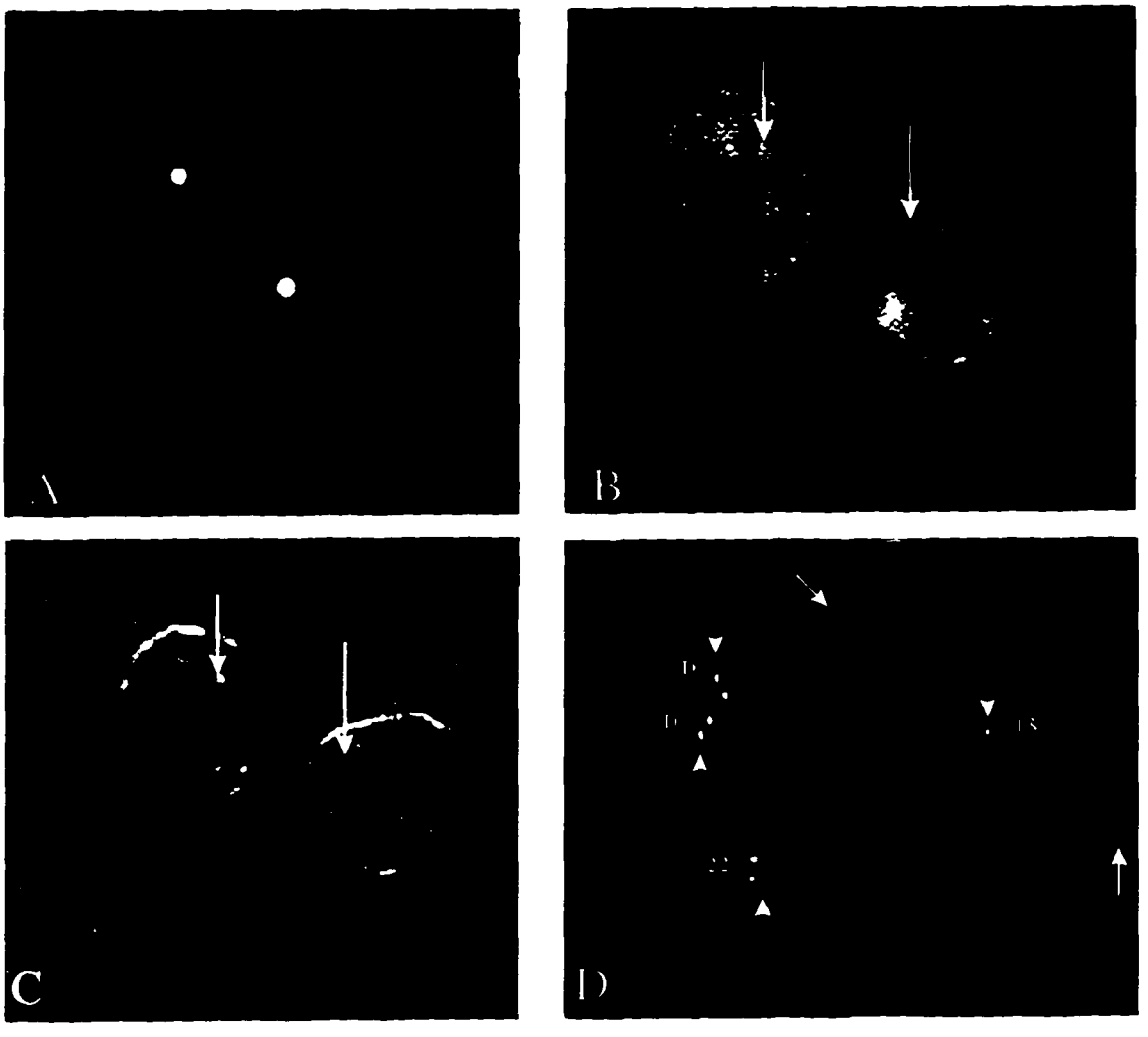
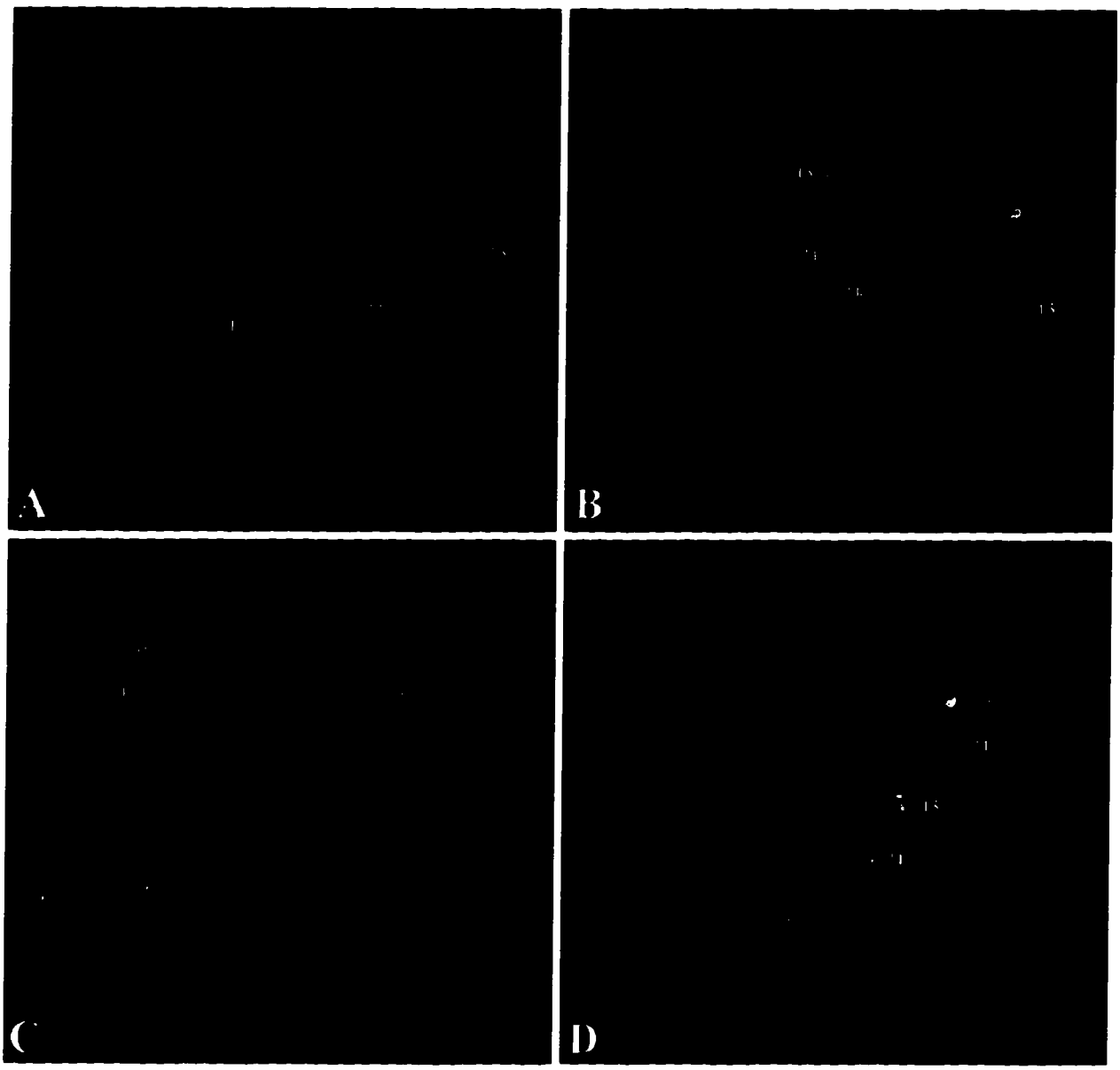


Fig. 5. Human chromosome spreads from phytohemagglutinin (PHA)-stimulated lymphocytes obtained from a normal male. **A, C** Chromosome spreads immunostained with Tau-1. **B, D** Chromosome spreads labeled with a cDNA probe to the centromeric DNA of chromosomes 13 and 21. *Numbers* indicate the various acrocentric chromosomes, while chromosomes 14 and 15 are labeled as **D** (see Materials and methods). Immunostaining patterns of Tau-1 on the NORs of mitotic chromosomes are consistent from spread to spread in the same individual



chromosome spreads were relocated, and individual chromosomes were identified (see Materials and methods). As can be seen (Fig. 5A, C), the same NORs exhibited the same patterns of intensity of fluorescence staining in each spread from the same individual. In this case, one of the 13s, one of the 22s and neither of the 21s showed Tau-1 staining while the other 13 and 22, as well as two of the D group chromosomes were stained with Tau-1. This consistent staining was confirmed in spreads from 50 cells. These results demonstrated that Tau-1 staining is consistent from one spread to the next in the same individual. Additionally, since various samples from the same individual were obtained longitudinally with consistent results, the data argued against artifacts that may be induced by culture conditions and sample preparation. Therefore, Tau-1 staining of NORs could conceivably be a heritable characteristic much like the silver-staining pattern of NOR proteins. However, heritability can only be established in multigenerational family studies.

Since tau apparently makes up only a small percentage of the total protein in the cell nucleus, a large sampling of cultured cells was needed to confirm the presence of tau biochemically. In previous studies it has been reported that nuclear tau in cultured cells was insoluble in SDS gel electrophoresis buffer and must first be extracted with formic acid prior to immunoblot analysis (Loomis et al. 1990). To meet this criterion human lymphoblasts which have nuclear tau and can be cultured in large quantities, were used. Immunoblots were probed with Tau-1 and Tau-5, which recognize epitopes at amino acids 189-207 and 210-241, respectively. These two antibodies have the lowest k_ds of all the tau antibodies and could detect the small amount of tau present in the nucleolus (unpublished observation). Immunoblots with Tau-1 displayed a ladder of

immunoreactive polypeptides ranging for M_r 4500 to 6600 (Fig. 6, lane 3). Immunoblots with Tau-5 showed two bands at approximately M_r 5500 and 6600 (Fig. 6, lane 2).

Correlation of Tau-1 Immunostaining with Silver Staining

Since the variability of Tau-1 staining of NORs appeared reminiscent of that observed with silver-staining methods used to detect NORs active in the preceding interphase (Goodpasture and Bloom 1975), studies were conducted to determine how Tau-1 staining patterns correlated with staining patterns from the silver-NOR procedure. This correlative analysis was possible since both Tau-1 staining and silver deposition on NORs are constant for individual chromosomes within a given patient. Ammoniacal silver stains ribonucleic acidic proteins around the NORs of acrocentric chromosomes that have been hypothesized to be involved in the binding of DNA to regulatory factors. As such, the amount of silver staining has been reported to be directly proportional to the transcriptional activity of a given rRNA gene cluster (Henderson et al. 1972). Chromosome spreads from lymphocytes isolated from the same individual as spreads in Fig. 5, were processed for silver staining, images of each spread were captured, their location recorded, and the spreads processed for FISH. The same spreads were relocated and the individual acrocentric chromosomes identified by FISH using cDNA probes to chromosomes 13/21 (Fig. 7A) and 15 (Fig. 7B). For the individual used for this study, chromosomes 21 and 22 as well as both pairs of 13 and 15 were silver stained (Fig. 7C). By comparison, some but not all of these chromosomes reacted with the Tau-1 monoclonal antibody (Fig. 5A, C; two D group chromosomes and nos. 13 and 22). This preliminary study suggests that tau staining on NORs was not equivalent to silver staining although, like silver staining, it was consistent from spread to spread within an individual.

Fig. 6. Immunoblot of isolated nuclei from human lymphoblasts probed with the monoclonal antibodies, Tau-1 and Tau-5. For a positive control, 120 ng of recombinant human tau expressed in *Escherichia coli* using the htau40 clone (Goedert et al. 1989) was loaded in lane 1, while lanes 2 and 3 contained 50 μg of formic acid-extracted protein from the isolated nuclei of human lymphoblasts. The samples were prepared as described in Materials and methods and separated on a 6%-12% SDS-polyacrylamide gradient gel. Proteins were transferred to nitrocellulose, and probed with Tau-5 (lanes 1 and 2) and Tau-1 (lane 3). Isolated nuclei from human lymphoblasts contain several polypeptides that can be stained with Tau-1 and Tau-5

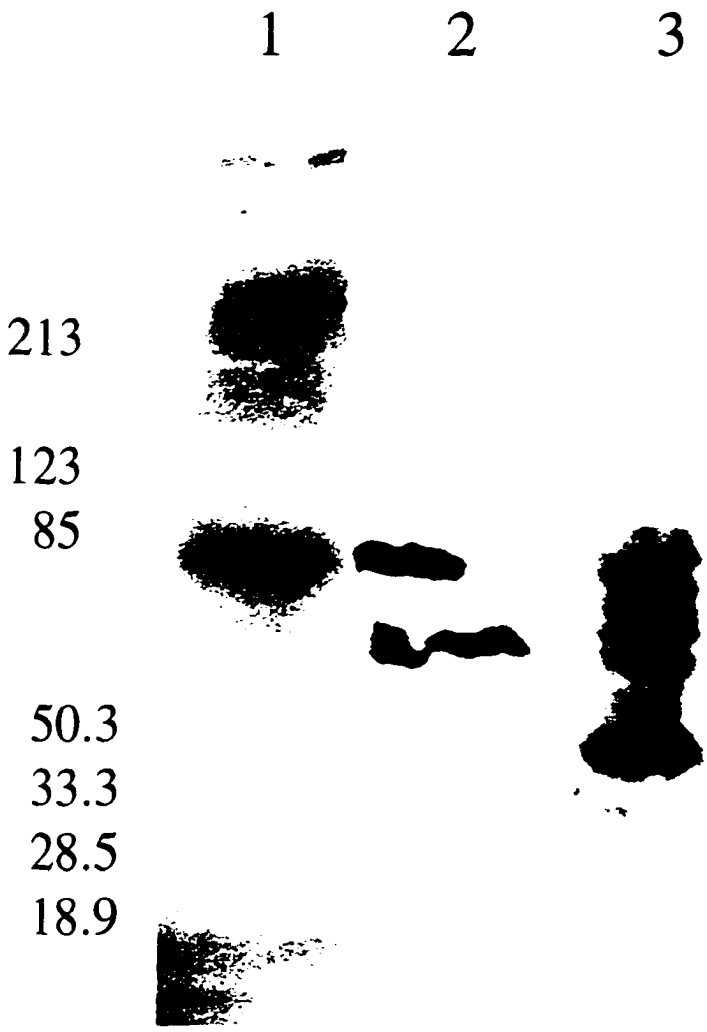
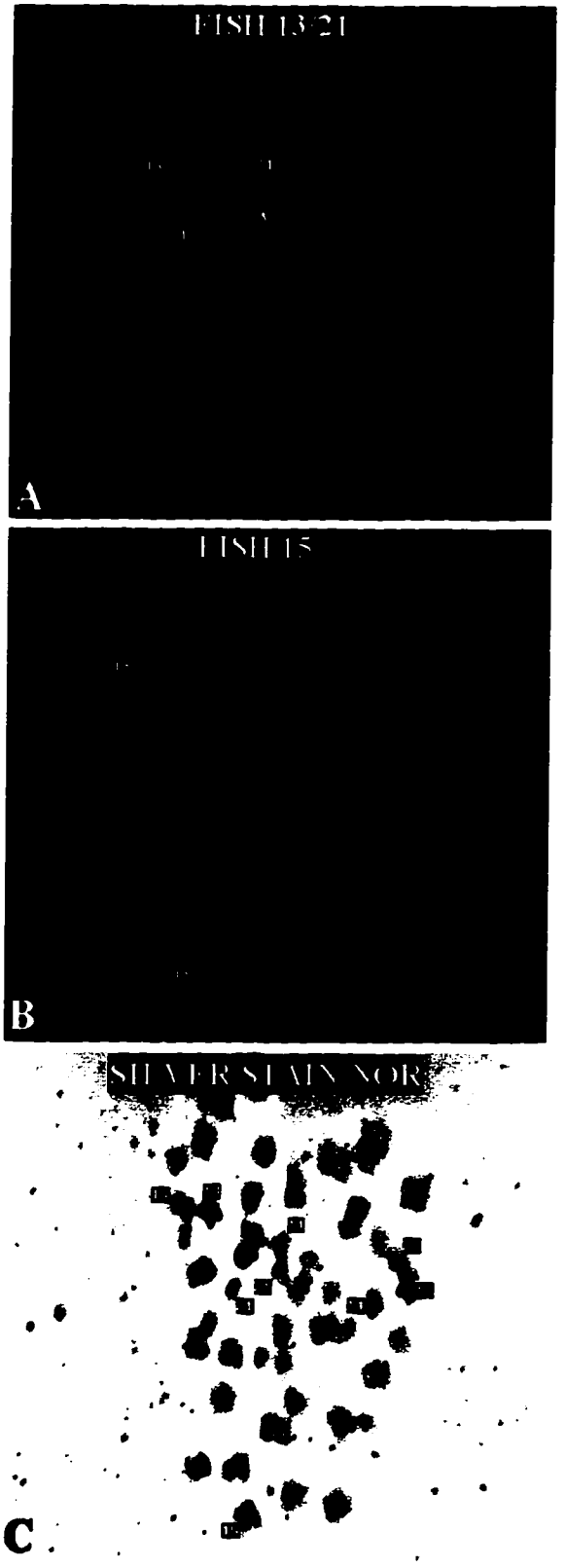


Fig. 7. Human chromosome spreads from a normal male processed for silver staining and FISH. **A** Chromosomes (*red*) identified with a cDNA probe that recognizes the centromeres of chromosomes 13 and 21 (*yellow*). **B** Chromosomes (pseudocolored *red*) with a cDNA probe to the centromere of chromosome 15 (pseudocolored *yellow*). **C** The same spread stained with ammoniacal silver nitrate



Quantitation of Nucleolar Tau in "Resting" Versus "Dividing" Cells

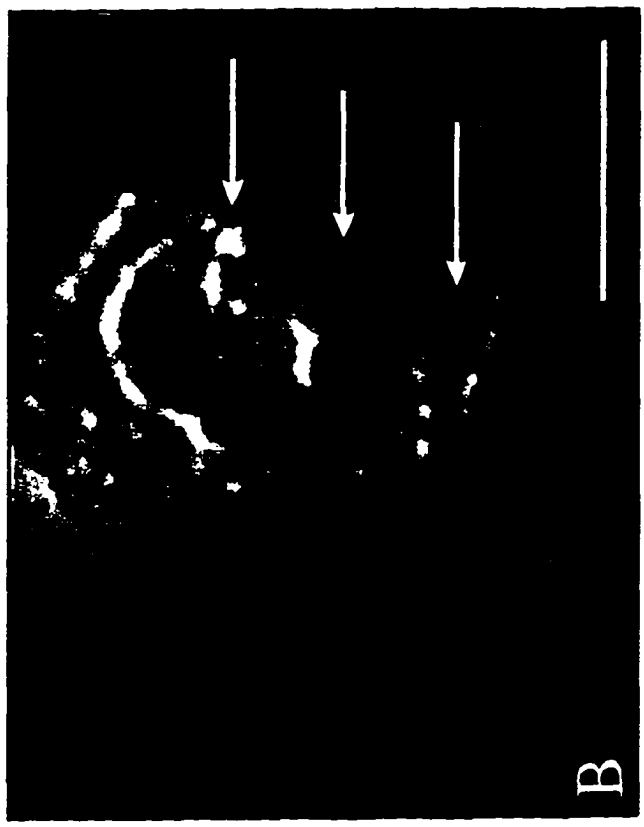
During these studies, differences between tau immunostaining intensities in transformed cells suggested that nuclear tau may be upregulated in transformed cell lines. To test this hypothesis, the overall staining intensity of nucleoli in dividing and nondividing cells was measured. The staining intensity in nucleoli of lymphocytes that were PHA stimulated (dividing) was compared with the staining intensity of nucleoli from lymphocytes that were not stimulated (nondividing). Images of nonstimulated lymphocytes showed nuclear tau localization to be confined only to a single small nucleolus by comparing Tau-1 immunofluorescence (Fig. 4A) with Hoechst and DIC images (Figs. 4B, C). However, Tau-1 immunostaining of PHA-stimulated lymphocytes revealed nuclear tau throughout an enlarged nucleolus or several nucleoli (arrows, Fig. 8A). The nucleolus or nucleoli are clearly observed by DIC optics in Fig. 8B (arrows). To determine whether nuclear tau was upregulated in dividing cells, total Tau-1 immunofluorescence intensity was measured for each nucleus. Quantitation of these nuclei demonstrated a significant increase in total Tau-1 immunofluorescence in PHA-stimulated cells from whole blood drawn from four individuals (Fig. 9). This suggested that nuclear tau is upregulated in cells as they are entering division.

DISCUSSION

Is Tau a Component of Nucleoli and NORs?

The Tau-1 monoclonal antibody is the only tau antibody to date shown to react with the fibrillar regions of nucleoli as well as the NORs of mitotic chromosomes in cultured cells (Loomis et al. 1990; Wang et al. 1993). All attempts to obtain similar results using other monoclonal antibodies have failed. However, whenever whole

Fig. 8. PHA-stimulated human lymphocytes immunostained with Tau-1. **A** Lymphocyte nucleus immunostained with Tau-1 and corresponding DIC image **B** *Arrows* identify Tau-1 staining in the nucleolus. PHA-stimulated lymphocytes immunostained with Tau-1 display a punctate staining pattern throughout the nucleus. Bar represents 10 μm



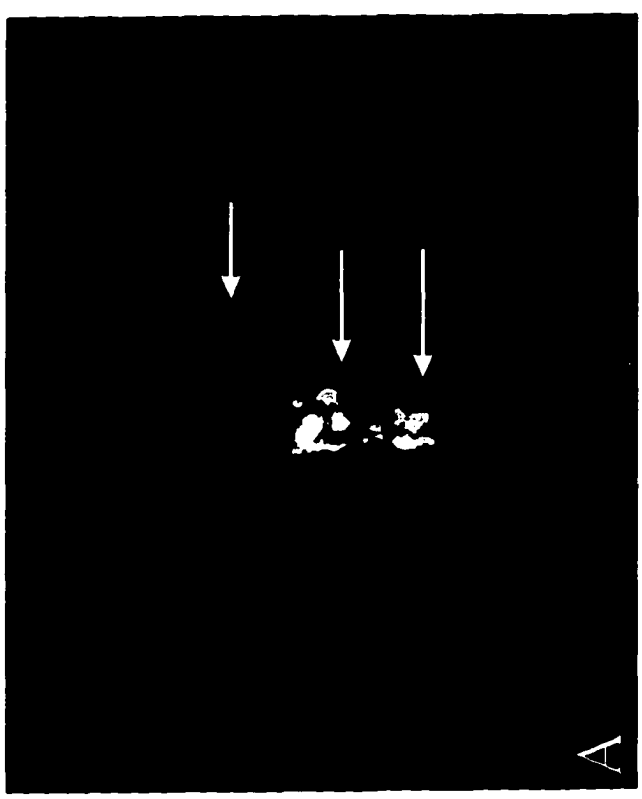
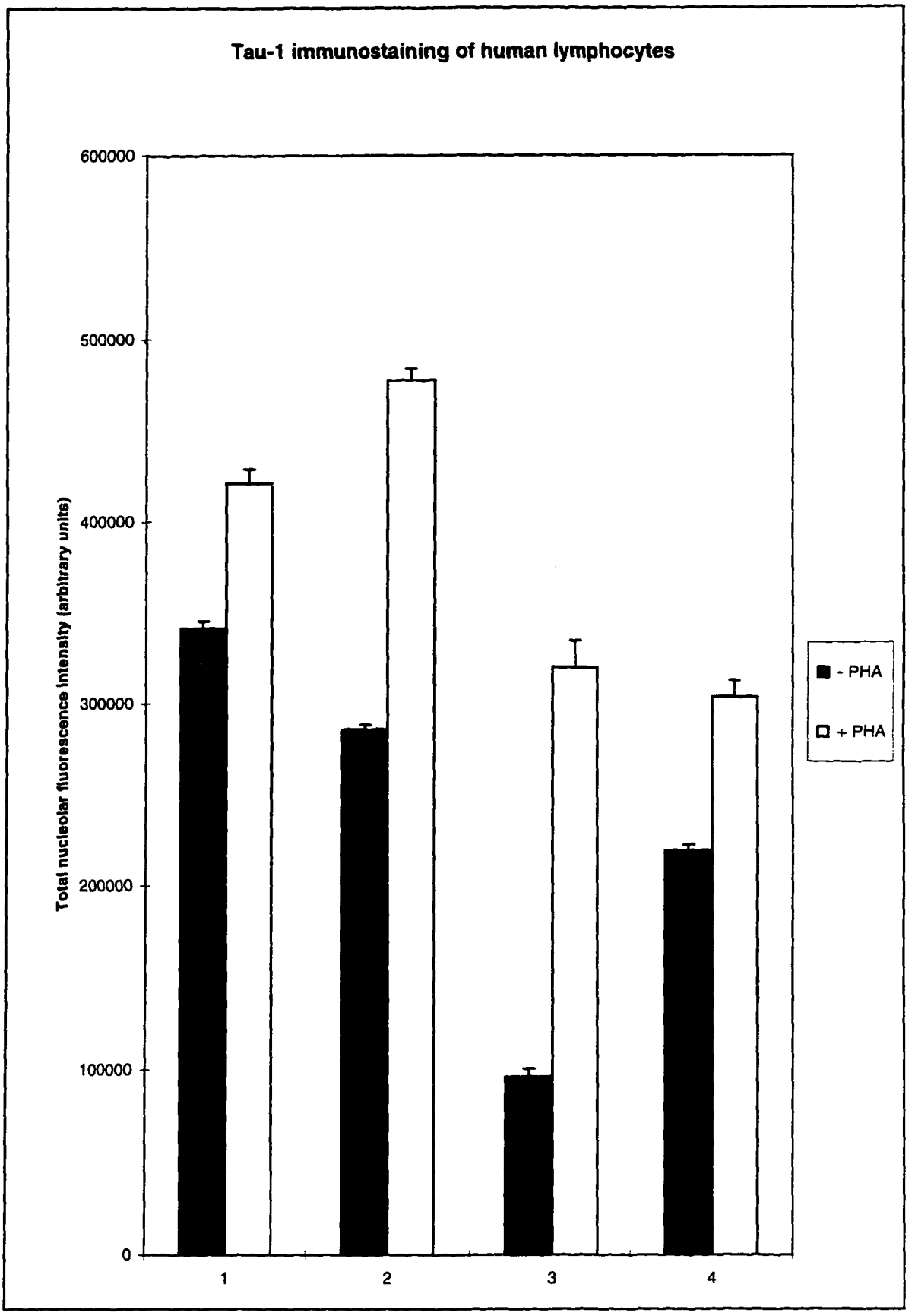


Fig. 9. Total fluorescence intensity of Tau-1 immunostained lymphocyte nucleoli. Each *set of bars* represents one sample of whole blood that was cultured for 72 h with or without PHA. *Error bars* indicate 1 SD (n=50)



primate cell nuclei have been isolated and subjected to immunoblot analyses, the entire tau molecule has been found within the confines of the nucleus. This was especially notable using CG neuroblastoma cells, which were shown to contain no discernible levels of cytoplasmic tau but tested positive for nuclear tau antibodies whose epitopes nearly spanned the tau molecule (Loomis et al. 1990). Furthermore, our laboratory has also reported the presence of tau immunofluorescence in nuclei isolated from frozen human brain using three monoclonal antibodies (Brady et al. 1995). In contrast to our report, the staining of brain nuclei was largely punctate and extranucleolar, although some nucleolar staining was occasionally observed with all three antibodies employed. Differences in immunolocalization patterns may reflect differences in cell cycle stage and/or cell differentiation. The lack of nucleolar tau in certain terminally differentiated cells, such as neurons, suggests its functions are no longer necessary and the small number of nuclei displaying nucleolar tau may be undergoing intense changes in rRNA synthesis (Brady et al. 1995). Moreover, confounding all attempts at efficacious tau immunolocalization is the extreme sensitivity of numerous tau epitopes to fixation conditions. Except in the disease stages (Papasozomenos 1995, see below), localization of tau to the nucleus or nucleolus in human brain has not been reported. It was not until nuclei purified from frozen human brain were subjected to the same fixation protocols as cells in culture that tau localization was observed in both the nucleus and nucleoli (Brady et al. 1995, see above). Additionally, the ability to demonstrate tau localization in oligodendrocytes was shown to be affected both by fixation and by the sequence specificity of the monoclonal tau antibody employed (LoPresti et al. 1995). Undoubtedly, the extreme flexibility of the

tau molecule explains this phenomenon as is discussed elsewhere (Brady et al. 1995; LoPresti et al. 1995).

Papasozomenos (1995) has localized tau immunoreactivity within neuronal nuclei in an autopsy case of presenile dementia with motor neuron disease. The staining was distributed throughout the nucleus in a diffuse manner and was excluded from the nucleolus. He hypothesized that tau may play a role in the stress response, acting similarly to heat shock proteins. Heat shock proteins are known to migrate transiently into the nucleolus, redistribute in the nucleus and exit into the cytoplasm following heat shock (Welch and Mizzen 1988). These proteins are also required for the transport of certain proteins into the nucleus (Shi and Thomas 1992). Since nuclear localization of the M_r 7200 stress-induced heat shock protein is excluded from the nucleoli of cells that have been heat shocked (Welch and Mizzen 1988), Papasozomenos (1995) suggested that this localization of tau to the nucleus was in response to chronic stress underlying the pathology of the disease.

Additional characterization of the ability of tau to associate with nuclear components was carried out by Lu and Wood (1993). They found that when fluorescently labeled bovine tau was microinjected into Chinese hamster ovary cells (CHO), which contained no tau mRNA or tau protein (Wang et al. 1993), the tagged protein localized to both the centrosome and to the nucleolus. Five to ten minutes after injection, staining of the centrosome was found to decrease with an accompanying detection of fluorescently labeled tau on the microtubule network. Unlike centrosomal staining, the strong nucleolar staining by tau was maintained throughout the entire experimental time period

indicating that it was targeted directly to the nucleolus where it functioned in a different capacity than cytoplasmic tau.

Here we report staining of interphase nucleoli and mitotic NORs in HeLa cells, nontransformed fibroblasts, and whole blood lymphocytes using the Tau-1 antibody. Again, no other tau monoclonal antibody stained these structures; however, immunoblots of formic acid-solubilized nucleoli obtained from lymphoblasts were positive for both Tau-1 and Tau-5 monoclonal antibodies, two antibodies that recognize distinct epitopes on authentic tau. The battery of antibodies used in previous immunoblot studies (Loomis et al. 1990) could not be used since only Tau-1 and Tau-5 have k_ds low enough to detect the small amounts of tau apparent in lymphoblast nuclei (unpublished observations). Therefore, the results obtained previously, and those described herein, continue to suggest strongly that Tau-1 immunofluorescence in interphase nuclei and on mitotic NORs is due to reaction with authentic tau.

mRNA Targeting and Tau Function

Recently, a 2 kb tau mRNA was discovered in our laboratory in neuroblastoma cells that may be involved in targeting tau to nonmicrotubule functions (Wang et al. 1993). This 2 kb mRNA appeared to be upregulated in human frontal cortex from AD patients (Goedert et al. 1988) and contained the entire coding region of tau (Wang et al. 1993). Additionally, it was shown that the 2 kb transcript did not result from further processing of the 6 kb transcript, but was derived from the original tau mRNA. The difference in size may be due to additional polyadenylation sites such as those recently found in the 3' untranslated region of tau DNA clones of rat lacking exons 2 and 3 (Sadot et al. 1994). One polyadenylation site was located between nucleotides 1359 and 1364

within intron 13/14, which would likely yield a transcript encoding the 2 kb species. The other polyadenylation site was located between nucleotides 4953 and 4958, probably resulting in the 6 kb species (Sadot et al. 1994). The 6 kb mRNA and its large 3' untranslated region appeared to be targeted to microtubules within the axon hillock region of neurons (Litman et al. 1993). The size of the tau mRNA present in nonneural cells (HeLas, fibroblasts or lymphocytes) has yet to be determined, although testis has been shown to contain a 2 kb transcript, and the tau produced appears to localize to the manchette, a microtubule-containing organelle involved in shaping the nucleus of spermatids during spermiogenesis (Ashman et al. 1992). It is possible that the 2 kb mRNA serves to target tau to different functional associations such as ribosomal biogenesis or transcription in much the same way as the 6 kb transcript targets tau to microtubules in the axon hillock.

NOR Tau and Silver Staining

Tau-1 staining was detected in the nucleolus of interphase lymphocytes and on the NORs of mitotic chromosomes. Interestingly, the staining varied from one NOR to the next but remained the same in each spread within an individual, much like silver-staining patterns of NOR proteins. Ammoniacal silver staining of mitotic chromosomes can determine NOR "activity", i. e., the rRNA gene clusters that were most transcriptionally active in the preceding interphase (Henderson et al. 1972). The more silver deposited, the more active the gene cluster. Silver staining is also a heritable characteristic in that the silver deposition on specific NORs is not only consistent within an individual but is also inherited (Markovic et al. 1978). Our results suggest that the intensity of Tau-1 staining of NORs on specific acrocentric chromosomes may be a heritable trait similar to silver

staining, although this remains to be tested in definitive family studies. However, Tau-1 antibody staining does not appear to correlate with silver staining of the NORs and is presumably indicative of some other functional state of the NOR.

The difference in Tau-1 staining patterns could indicate that tau is not a silver-staining protein in situ, or that it functions differently than most silver-staining proteins. One example of a silver-staining protein is nucleolin, which is known to have an amino-terminal half containing several repeating sequences rich in glutamic and aspartic acid, much like the MAP-binding domain of tubulin (Mamrack et al. 1979). Nucleolin is also characterized by phosphorylated serine residues and a carboxy-terminus rich in glycine with modified arginine residues (Caizergues-Ferrer et al. 1989). The acidic domain of silver-staining proteins could possibly interact with histones to relax the ribosomal chromatin while the carboxy-terminus would apppear capable of binding rRNA transcripts (Sommerville 1986). Tau does contain an acidic amino-terminus although not nearly as acidic as nucleolin (Sommerville 1986); thus, it could conceivably interact with histones. However, the microtubule-binding domain on tau is extremely basic suggesting that it could bind to the acid region of nucleolin and/or the rDNA, itself. Silver-staining proteins have been thought to be involved in transcription (Howell 1982; Jordan 1987). Since tau has been localized to both the NORs and to ribosomes, it could be involved in protein synthesis or ribosomal biogenesis, either by itself or by interaction with nucleolin or other nuclear proteins to assist in transcriptional control of ribosomal genes.

Tau in PHA-stimulated Lymphocytes

Preliminary evidence has suggested that transformed lymphocytes stained more intensely with Tau-1 than those freshly isolated from blood. We reasoned that this was

due to cell cycle differences between whole blood and immortalized lymphocytes. To determine whether this was the case, Tau-1 staining intensity of nucleoli was measured in PHA-stimulated lymphocytes and in nonstimulated lymphocytes. PHA is a mucoprotein extracted from the red kidney bean, *Phaseolus vulgaris*, which causes an increase in RNA synthesis in cells in vitro after a culture lag of 24 h. During the next 24 h the nucleus enlarges and DNA synthesis begins (Rooney and Czepulkowski 1986). Quantification of staining intensity showed an increase in total Tau-1 immuno-fluorescence in PHA-stimulated cells. Upregulation of total nuclear tau would be expected in a cell stimulated to divide and in need of an increased rRNA output if tau were involved in some aspect of rRNA synthesis, processing, or transport. As the cell prepares to undergo mitosis, interactions with histones would occur throughout the nucleolus to relax ribosomal chromatin. Involvement of tau in these interactions could explain its localization throughout the nucleolus.

In summary, this work establishes tau localization outside of the central nervous system and reinforces the likelihood that tau functions, in some manner, as a nucleolar protein. We have shown that Tau-1 immunoreactivity is found associated with the NOR regions of acrocentric chromosomes, the site of the rRNA genes, and the fibrillar regions of the nucleoli, the site of rRNA transcription in human nonneuronal cells. We have also presented evidence indicating that Tau-1 staining may be a heritable characteristic similar to silver staining, which is associated with transcriptionally active rRNA genes. This lends further support to the hypothesis that nuclear tau may be involved in protein biosynthesis or ribosomal biogenesis not just in neural cell lines but throughout the organism.

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NUCLEOLAR TAU IN ALZHEIMER'S DISEASE LYMPHOCYTES

by

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ABSTRACT

Tau is a neuronal microtubule-associated protein which localizes primarily in the axons and somatodendritic compartment of neurons (Binder et al. 1985; Papasozomenos and Binder 1987), but has also been found to be associated with nucleoli and nucleolar organizer region of lymphocytes (Thurston et al. 1996). Recent evidence from our laboratory (Thurston et al. 1996) has suggested that transformed lymphocytes stain more intensely with Tau-1 than those freshly isolated from whole blood. To confirm that the increase in Tau-1 staining during PHA stimulation was due to an increase in tau and not to a dephosphorylation event at the Tau-1 site, staining intensity of alkaline phosphatase treated PHA-stimulated lymphocytes was quantitated to determine if the increase in Tau-1 immunoreactivity was likely due to upregulation of tau protein rather than an unmasking of the Tau-1 epitope. Since nuclear tau also had properties similar to PHF-tau in AD, the staining intensity of Tau-1 in lymphocytes of AD patients was assayed, as well. No significant difference in total Tau-1 immunofluorescence was observed in PHA-stimulated or nonstimulated lymphocytes obtained from the blood of putative AD or age-matched control patients.

INTRODUCTION

Previously, it was demonstrated that the tau monoclonal antibody, Tau-1, stained the fibrillar regions of interphase nucleoli and at the nucleolar organizer regions (NORs) of acrocentric chromosomes of dividing primate cells in culture (Loomis et al. 1990; Wang et al. 1993, Thurston et al. 1996). Nucleoli are the sites of ribosome biogenesis while the NORs on the short arms of human acrocentric chromosomes (nos. 13, 14, 15, 21, and 22) represent the chromosomal sites of rRNA genes. The Tau-1 monoclonal

antibody (Loomis et al. 1990) recognized an epitope at amino acids 189-207 on the longest human tau isoform (Kosik et al. 1988). When this epitope was phosphorylated at Ser^{199} and Ser^{202}, as it was in AD, Tau-1 binding was occluded (Liu et al. 1993). Moreover, tau has been demonstrated in nuclei isolated from fresh frozen human brain (Brady et al. 1995). Interestingly, nuclear tau had solubility properties similar to PHF tau which had been found in both perinuclear and intranuclear locations (Metuzals et al. 1988). Nuclear tau is insoluble in SDS sample buffer, requiring formic acid extraction for solubilization, like some PHF tau. Unlike PHF tau, nuclear tau reacted robustly with Tau-1, indicating that it was not phosphorylated at Ser^{199} and Ser^{202} (Loomis et al. 1990).

Recent evidence has suggested that transformed lymphocytes stained more intensely with Tau-1 than those freshly isolated from whole blood (Thurston et al. 1996). Quantification of staining intensity showed an increase in total Tau-1 immuno-fluorescence in PHA stimulated cells as compared to nonstimulated lymphocytes. Upregulation of total nucleolar tau would be expected in a cell stimulated to divide and in need of an increased rRNA output if tau were involved in some aspect of rRNA synthesis, processing, or transport. The increase in Tau-1 staining as a result of PHA stimulation appeared to be due to an increase in tau and not a dephosphorylation event at the Tau-1 site, since treatment with alkaline phosphatase did not alter the nucleolar staining intensity.

Since nuclear tau also has properties similar to PHF tau in AD, we wished to look at the staining intensity of Tau-1 in lymphocytes of AD patients, as well. Furthermore, recent evidence indicated a decrease in the frequency of silver-stained NORs and satellite associations in lymphocytes from AD patients (Payao et al. 1994). Although Tau-1

staining did not correlate with silver staining in humans, it was a NOR-associated protein in lymphocytes (Thurston et al. 1996). Therefore, we compared Tau-1 staining intensity of nonstimulated lymphocytes in individuals with a putative diagnosis of AD with the staining intensity seen in age-matched controls. We also measured staining intensity in PHA-stimulated lymphocytes from AD patients and from controls. Quantification of staining intensity showed no consistent difference in total Tau-1 immunofluorescence in nonstimulated lymphocytes between AD or age-matched controls, as well as no significant difference in staining intensity in PHA-stimulated lymphocytes between AD or age-matched controls.

MATERIALS AND METHODS

Cell Culture

Fresh human lymphocytes were isolated from heparinized whole blood of putative Alzheimer's patients and age-matched controls. Lymphocytes were separated by Ficoll and cultured in RPMI 1640 (BioWhittaker) supplemented with 20% fetal bovine serum. For phytohemaglutinin (PHA) stimulation experiments, cultures were supplemented with 0.2 μg/ml PHA (GIBCO, Gaithersburg, MD) for 72 h. All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Immunofluorescence

Cells were cytocentrifuged onto glass microscope slides using a Shandon Cytospin III (Shandon Southern Instruments Inc., Sewickley, PA) at 500 rpm for 2 min. Subsequently, the cells were fixed in 3% ultrapure TEM grade formaldehyde (Tousimis Research Corp., Rockville, MD) in 100 mM TRIS-HCl, pH 7.4 (Buffer A), for 30 min at 24°C, and permeabilized in 0.5% Triton-X-100 in Buffer A for 5 min. For alkaline

phosphatase experiments, the cells were fixed, blocked with 2% BSA in Buffer A for 10 min, rinsed in Buffer A, and then incubated in Buffer A containing 0.5-1 units/ml of alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and 1 mM phenyl-methylsulfonyl fluoride (Sigma, St. Louis, MO) for 2 h at 37°C. After rinsing twice for 5 min in Buffer A, the attached cells were reduced in 0.5 μg/ml $NaBH_4$ in Buffer A for 4 min and processed for indirect immunofluorescence microscopy (Brinkley et al. 1988). The cell preparations were counterstained with 1 μg/ml of the DNA stain Hoechst 33258 (Sigma), rinsed in Buffer A, and coverslipped with 0.1% *p*-phenylenediamine (Sigma) in a 9:1 mixture of PBS:glycerol.

Quantitation of Immunofluorescence

After the slides were processed for immunofluorescence, video images of nucleoli were captured and stored on the computer. Samples processed for immunofluorescence were analyzed using a Jenalumar epifluorescence microscope equipped with differential interference contrast (DIC) optics and an Optronics Tec-470 CCD video camera (Goleta, CA) coupled to an Image-1 (Universal Imaging) image analysis system and an IBM compatible PC 486/33. Image processing was performed by setting the shutter speed and contrast controls on the camera as well as the gain and black level on the imaging program to provide an optimal signal. Then, 128 video images were summed into the foreground frame buffer, and a previously obtained background image was subtracted from the foreground buffer to control for uneven illumination. Image quantification was performed by thresholding the area to be quantitated and subsequently measuring the gray level of each pixel. Each pixel has a gray scale that ranges from 0 to 255. The pixels of each thresholded area were summed and the final measurement was recorded to the hard

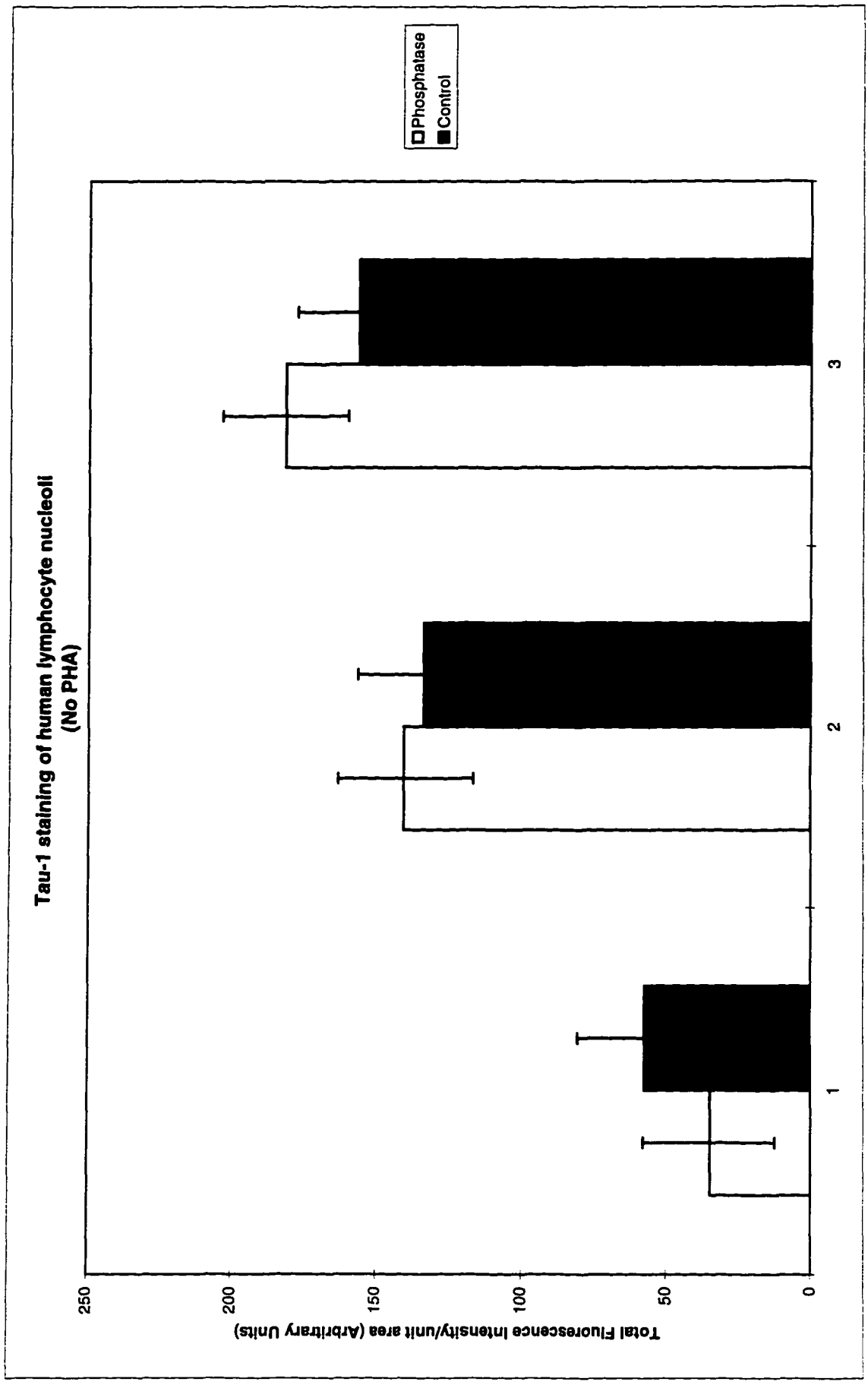
drive. The resultant data were imported into Excel, and fluorescence intensity for each nucleolus was analyzed. The nuclei from 50 cells were assayed per individual blood sample and the results expressed as average fluorescence intensity $\pm$ 1 standard deviation.

RESULTS

Quantitation of Nucleolar Tau in Alkaline Phosphatase treated lymphocytes

In previous studies (Thurston et al. 1996), differences between tau immunostaining intensities in PHA-stimulated lymphocytes and nonstimulated cells suggested that nucleolar tau is upregulated by PHA stimulation. The Tau-1 monoclonal antibody is the only tau antibody to date to react with nucleoli and NORs by immuno-fluorescence; therefore, Tau-1 staining intensity was used. Since the Tau-1 epitope can be masked by phosphorylation, experiments were performed to determine whether the increase in Tau-1 staining was due to an increase in tau or due to a dephosphorylation event unmasking the Tau-1 site. Lymphocytes from fresh whole blood were isolated from three normal individuals, split into two aliquots, and either treated with alkaline phosphatase or with buffer alone. Fifty cells from each individual were measured for average nucleolar Tau-1 immunofluoresecence intensity and compared with nontreated lymphocyte nuclei. Quantitation of these nucleoli demonstrated no significant increase in total Tau-1 intensity in alkaline phosphatase treated cells within any one individual (Fig. 1). During PHA-stimulation, Tau-1 staining intensity is significantly increased. Again, to confirm that this increase is due to an increase in tau and not due to a dephosphorylation event at the Tau-1 site, we isolated lymphocytes from three normal controls, stimulated the cells with PHA for 72 h, and treated half of the cells with alkaline phosphatase and the other half in buffer alone. When total Tau-1 immuno-fluorescence

Fig. 1. Total fluorescence intensity of Tau-1 immunostained nucleoli in lymphocytes cultured from three normal individuals and treated with alkaline phosphatase. Each *set of bars* represents alkaline phosphatase treated or untreated lymphocytes from a normal control. *Error bars* indicate one standard deviation (n=50)



intensity was measured for each nucleoli and the values averaged, there was no consistent increase in total Tau-1 immunofluorescence in PHA-stimulated cells treated with alkaline phosphatase, as compared to nontreated PHA-stimulated lymphocytes (Fig. 2). In fact, in one instance, phosphatase treatment resulted in a lower Tau-1 signal. Hence, an upregulation of tau is likely the cause of the increased Tau-1 immunofluorescence rather than an unmasking of phosphoserines in the Tau-1 site.

Quantitation of Nucleolar Tau in Nonstimulated Lymphocytes from AD patients

Since nucleolar tau also has properties similar to PHF-tau in AD (Loomis et al. 1990; Wang et al. 1993), we wished to look at the staining intensity of Tau-1 in lymphocytes of AD patients, as well. Lymphocytes were isolated from six patients with putative AD and six age-matched controls, cultured for 72 h, and processed for Tau-1 immunofluorescence. Fifty cells from each individual were measured and the Tau-1 staining intensity value averaged. No significant difference in total Tau-1 immunofluorescence between lymphocytes from putative AD patients or age-matched controls was noted (Fig. 3).

Quantitation of Nucleolar Tau in PHA-stimulated Lymphocytes from AD patients

The staining intensity of Tau-1 in the nucleoli of PHA-stimulated lymphocytes of AD patients was compared to PHA-stimulated lymphocytes from age-matched controls in order to determine if nucleolar tau levels were differentially affected by lectin treatment in lymphocytes obtained from whole blood of individuals with Alzheimer's disease. Lymphocytes were isolated from three putative AD patients and three age-matched controls, treated with PHA for 72 h, and then processed for Tau-1 immunoflourescence. Fifty cells per group were quantified for total nucleolar Tau-1 staining intensity; again, no

Fig. 2. Total fluorescence intensity of Tau-1 in nucleoli of lymphocytes from three normal individuals, cultured in PHA and treated with alkaline phosphatase. Each *set of bars* represents lymphocytes from a normal control treated with alkaline phosphatase or untreated. *Error bars* indicate one standard deviation (n=50)

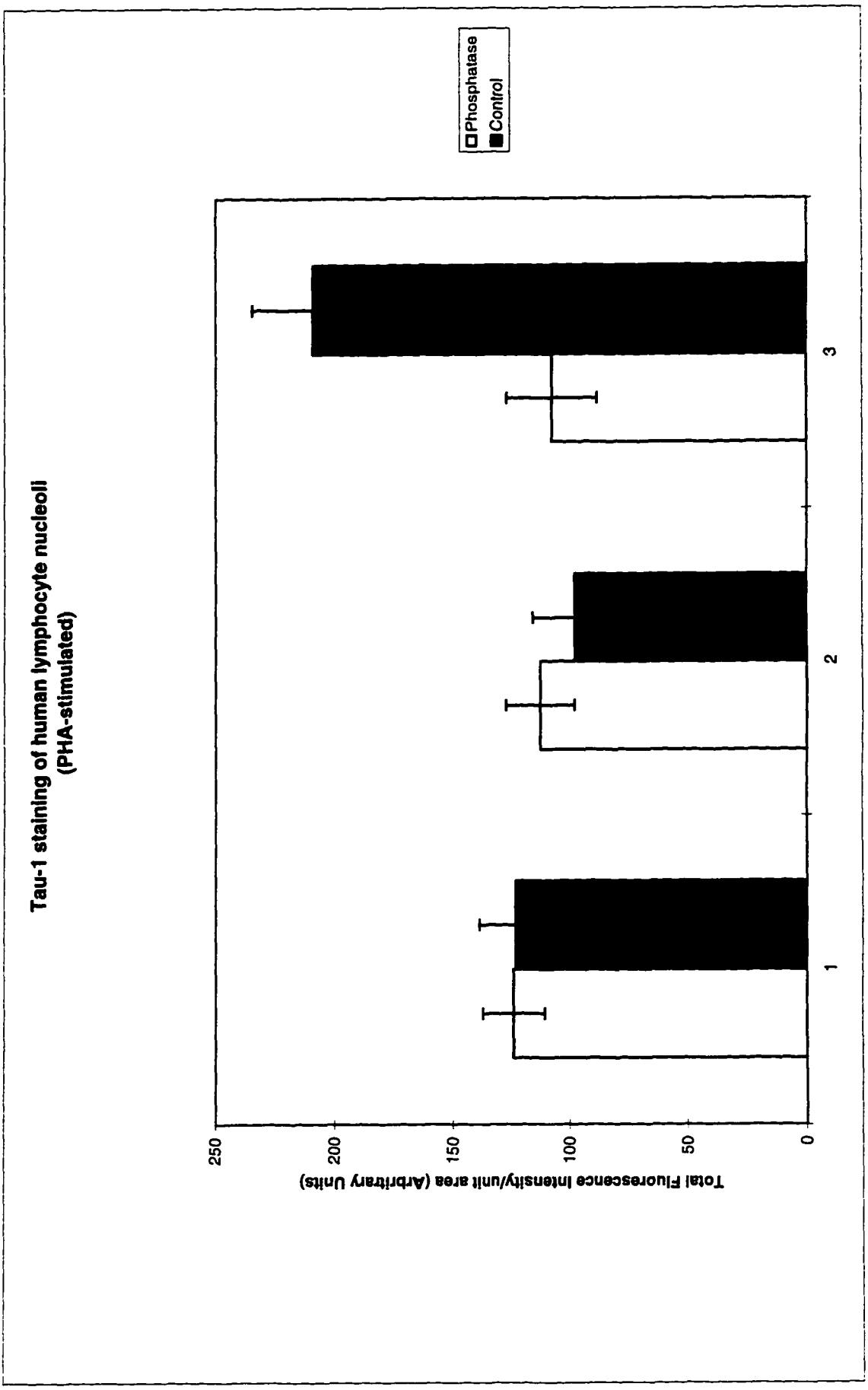
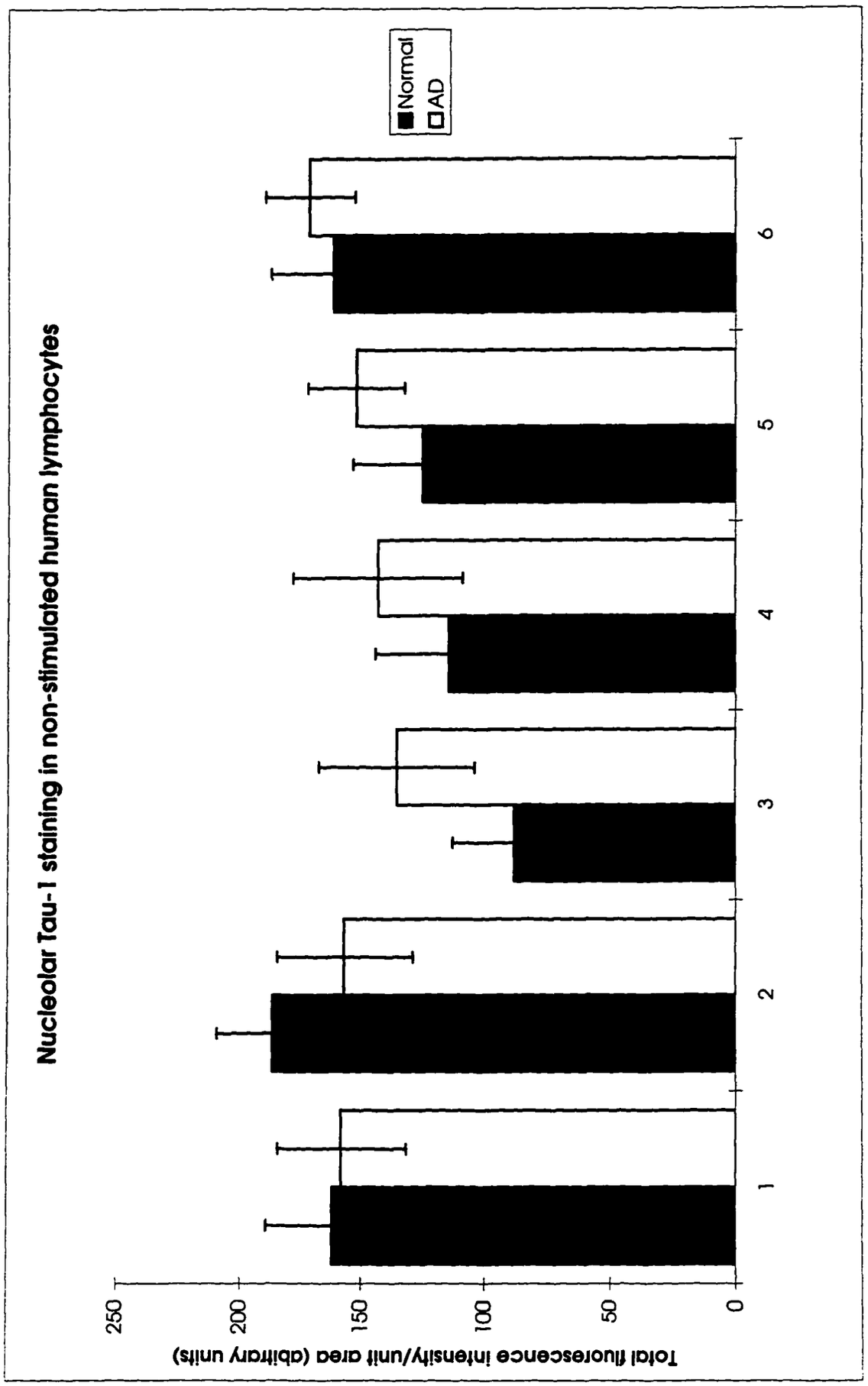


Fig. 3. Total fluorescence intensity of Tau-1 in nucleoli in lymphocytes cultured from six putative Alzheimer patients and six age-matched controls. Each *set of bars* represents lymphocyte nucleoli from an age-matched control or a putative Alzheimer's diseased individual quantified on the same day. *Error bars* indicate one standard deviation (n=50)



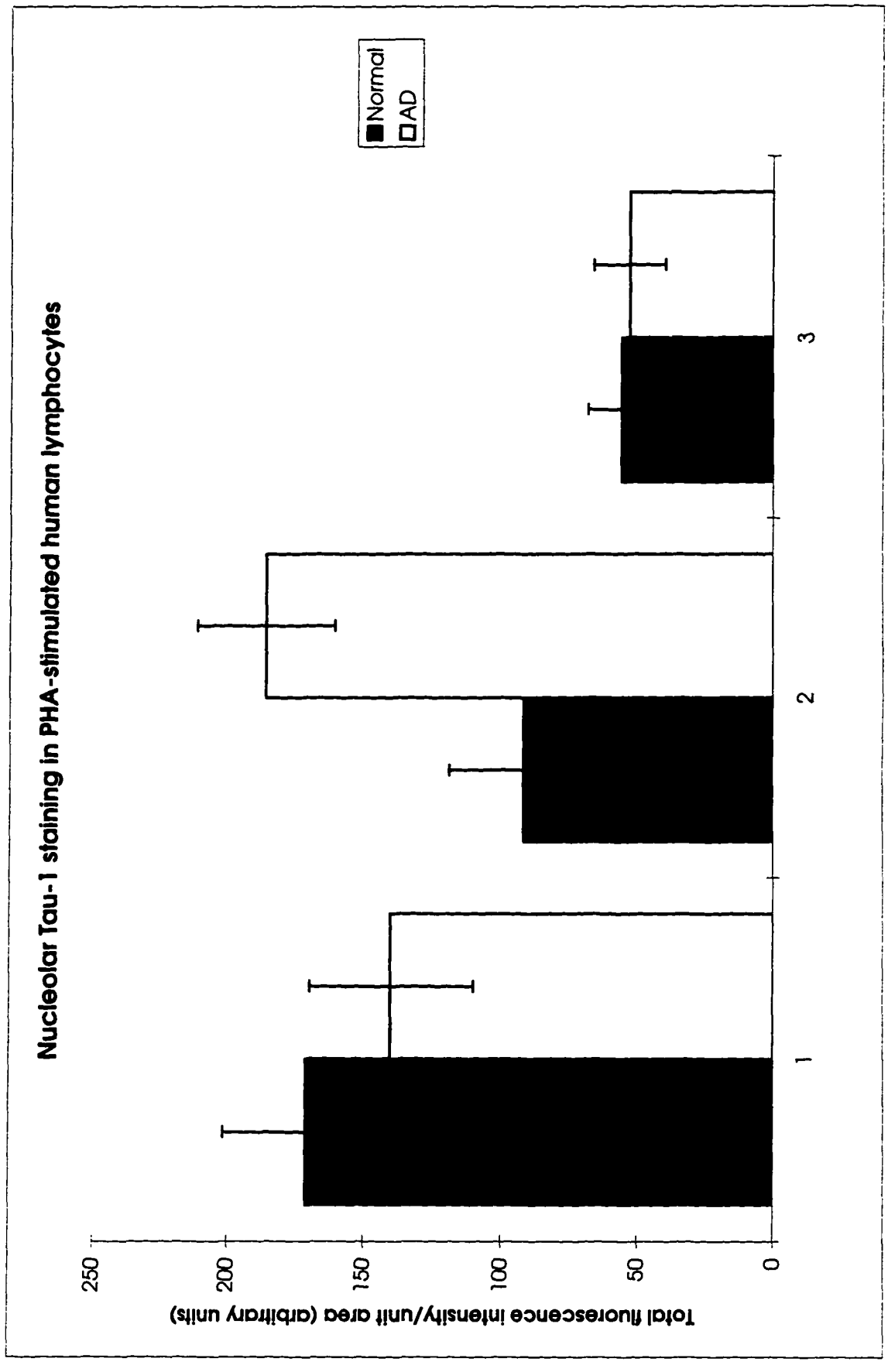
consistent difference in average Tau-1 immunofluorescence was observed between groups of PHA-stimulated lymphocytes from AD patients or controls (Fig. 4).

DISCUSSION

Tau in PHA-stimulated Lymphocytes

From previous studies in our laboratory, differences between tau immunostaining intensities in transformed cells suggested that nucleolar tau may be upregulated in transformed cell lines. To determine if this was the case, Thurston et al. (1996) compared the staining intensity in nucleoli of lymphocytes that were PHA stimulated (dividing) to the staining intensity of nucleoli from lymphocytes that were not stimulated (nondividing). Images of nonstimulated lymphocytes showed nuclear tau localization to be confined only to a single small nucleolus by Tau-1 immunostaining. However, PHA-stimulated lymphocytes displayed nuclear tau throughout an enlarged nucleolus or several nucleoli. Quantitation of these nuclei demonstrated a significant increase in total Tau-1 immunofluorescence in PHA-stimulated cells from whole blood. This suggested that nucleolar tau was upregulated in cells as they were entering division. Upregulation of total nucleolar tau would be expected in a cell stimulated to divide and in need of an increased rRNA output if this tau was involved in some aspect of rRNA synthesis, processing, or transport. Here, we quantitated total Tau-1 staining intensity in PHA-stimulated lymphocytes treated with alkaline phosphatase to determine if the increase in staining was due to an increase in tau or a phosphorylation event at the Tau-1 epitope. Our results suggested that tau was actually increased in dividing lymphocytes and that increased staining as the result of PHA stimulation was not due to a dephosphorylation event at the Tau-1 site.

Fig. 4. Total nucleolar Tau-1 fluorescence intensity in PHA-stimulated lymphocytes from three putative Alzheimer's patients and three age-matched controls. Each *set of bars* represents lymphocyte nucleoli from an age-matched control or a putative Alzheimer's diseased individual quantified on the same day. *Error bars* indicate one standard deviation (n=50)



Nuclear tau resists solubilization by conventional methods and must first be extracted with formic acid prior to solubilization in SDS-electrophoresis sample buffer, properties similar to tau in certain isolates of PHF. Hence, we wished to look at the staining intensity of Tau-1 in lymphocytes of AD patients versus control in order to ascertain whether tau alterations were mirrored in nuclei of peripheral blood cells. No consistent alterations were observed, suggesting that nucleolar tau was not involved in the AD process, at least in peripheral blood. In addition to these studies, Brady et al. (1995) documented the existence of nuclear tau in nuclei isolated from brain. They quantitated nuclear tau isolated from AD and control brains but were not able to demonstrate consistently altered protein levels that would suggest an involvement in AD.

Previous studies of Alzheimer's disease involving peripheral blood cells have shown no consistent alterations in AD versus age-matched controls. Calcium concentrations of basal and PHA-simulated lymphocytes for AD patients were significantly higher than in those of healthy age-matched controls, but not significantly higher than depressed patient controls (Bondy et al. 1996). They also showed that the kinetics of the calcium signal seemed to be altered in AD patients. The initial PHA-induced peak seemed to be delayed compared with healthy controls and depressed patients, although there was some overlap in both categories. Eckert et al. (1996) found no differences in calcium regulation between AD patients and age-matched controls in either basal or PHA-induced levels. Blood cell profile differences were similarly unremarkable: the number of neutrophils was similar in young, aged, and AD groups, although the number of lymphocytes decreased with age, independent of disease (Collins et al. 1991; MacDonald et al. 1982).

Our preliminary studies were intended to measure the Tau-1 staining intensity on individual NORs, but due to the abolishment of the Tau-1 site by standard cytogenetic fixation protocols, we were unable to obtain usable metaphase spreads. Instead, it was decided to quantitate total Tau-1 staining intensity of lymphocyte nucleoli, and no significant difference between PHA-stimulated lymphocytes from AD patients or controls was found. This is in contrast to work demonstrating a decrease in NOR silver staining and satellite association (SA) in PHA-stimulated lymphocytes from AD patients (Payao et al. 1994). A significant decrease in the frequency of SA and silver staining of chromosome 21 in their AD group was also noted, leading them to suggest a reduction in the activity of ribosomal genes in AD, specifically in chromosome 21. Although nucleolar tau did not appear to be a silver-staining protein, it was localized to the NORs and was upregulated in dividing cells. Decreased activity of ribosomal genes may be due to changes in the regulation and expression of the rRNA genes, which could result in the reduction of protein synthesis.

Lymphocytes from AD patients have been shown to be less viable than those of normal age-matched controls following X-irradiation (Robbins et al. 1983). Tobi et al. (1990) demonstrated a significant increase in the frequency of dicentric chromosomes when compared to normal controls after 3 Gy • -irradiation. There have also been a number of reports assessing the distribution of chromosome breakpoints in lymphocytes following irradiation, but no consistent pattern has emerged (for reviews, see Tawn et al. 1985). Irradiated lymphocytes from Down's Syndrome (DS) individuals, where many patients reaching middle age show neuropathological characteristics of AD, have also

shown a similar increase in the frequency of induced dicentrics (for review, see Tobi et al. 1990).

Interestingly, not only do individuals with DS or trisomy 21 often develop AD later in life, but in one inherited form of AD, families exhibit a higher incidence of trisomy 21 offspring (Heston et al. 1981; Heyman et al. 1983a,b; Heston 1984; Cutler and Heston 1985; Potter 1991). Many cases of DS are due to nondisjunction of the acrocentric chromosome 21. The acrocentric chromosomes (nos. 13, 14, 15, 21, and 22) are prone to nondisjunction resulting in trisomy, and many investigators have suggested that this was due to aggregation and cohesiveness at their NOR regions during metaphase (Markovic et al. 1978; Hansson 1979; Mirre et al. 1980; Jackson-Cooke et al. 1985; Jones et al. 1988). In fact, Jackson-Cooke et al. (1985) noted that in some cases of trisomy 21, double NORs were seen in the parent in which the nondisjunction occurred. It was known that the rRNA transcriptional activity, the intensity of silver staining, and the frequency of chromosomal associations were positively correlated (Schmid et al. 1974; Zankl and Zang 1974; Warburton et al. 1976; Hayata et al. 1977; Miller et al. 1977). Furthermore, studies have shown that active NORs were observed and often physically associated throughout most of meiosis I when most cases of nondisjunction involving trisomy 21 occur (Mirre et al. 1980; Schmid et al. 1974; Zankl and Zang 1974; Warburton et al. 1976; Hayata et al. 1977; Miller et al. 1977). Young mothers of Down's Syndrome children found to be at increased risk for developing Alzheimer's disease have been postulated to be mosaic for trisomy 21 potentially produced by somatic nondisjunction (Avramopoulos et al. 1996). The presence of tau, the neurofibrillary tangle protein, on acrocentric chromosomes, the possible site of nondisjunction, and its involvement with

Alzheimer's disease make it an interesting potential molecular link between AD and DS. Further work is needed in this area to determine if nucleolar tau has a role in Alzheimer's disease.

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NUCLEOLAR LOCALIZATION OF THE MICROTUBULE-ASSOCIATED PROTEIN TAU IN NEUROBLASTOMAS USING SENSE AND ANTI-SENSE TRANSFECTION STRATEGIES

by

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ABSTRACT

The Tau-1 monoclonal antibody was localized to the nucleolus of interphase cells and the nucleolar organizing regions (NORs) of acrocentric chromosomes in cultured human cells. Putative nucleolar and NOR tau was found in CG neuroblastoma cells which contain nucleolar tau and little cytoplasmic tau. To establish further the presence of tau in the nucleolus of these cells, sense and anti-sense transfection strategies were used. CG neuroblastoma cells were transfected with tau sense cDNA and immunostained with Tau-1. Cytoplasmic Tau-1 staining was greatly increased in CG cells which contain very little endogenous cytoplasmic tau. Nucleolar Tau-1 staining was also increased in certain CG cells, indicating an increase in nucleolar tau in a subset of transfected cells. CG cells were also transfected with tau anti-sense cDNA which abolished Tau-1 staining in the nucleolus. These results contribute to a growing body of evidence defining tau as a multifunctional protein found in both the cytoplasm and nucleoli of neuronal cells.

INTRODUCTION

Tau has been found to be a neuronal microtubule-associated protein (MAP) which localizes primarily in the axon (Binder et al. 1985; Kowall and Kosik 1987; Brion et al. 1988; Trowjanowski et al. 1989) and exists in many isoforms as a result of alternative splicing (Himmler 1989; Himmler et al. 1989; Goedert et al. 1989; Andreadis et al. 1992) and phosphorylation (Cleveland et al. 1977). Although tau was originally described as a MAP (Cleveland et al. 1977), it has also been found associated with ribosome (Papasozomenos and Binder 1987) and in the nucleus (Brady et al. 1995; Loomis et al. 1990; Wang et al. 1993; Lu and Wood 1993; Lambert et al. 1995; Greenwood and Johnson 1995; Thurston et al. 1996). The functional significance of ribosomal or nuclear

tau is as yet unclear; however, by Tau-1 immunofluorescence, nuclear tau specifically localizes to the fibrillar regions of interphase nucleoli and the nucleolar organizer regions of mitotic chromosomes (Brady et al. 1995; Loomis et al. 1990; Wang et al. 1993; Thurston et al. 1996), suggesting a functional role for tau in the nucleus.

Structurally, tau is a tripartite phosphoprotein composed of a variable N-terminal domain, a constant central domain, and a microtubule-binding domain in the C-terminal region. In the adult human brain, six isoforms have been found with derived molecular weights of 37 to 46 kDa and ranging in size from 352 to 441 amino acids which result from alternative splicing of a single mRNA transcript (Lee et al. 1988; Himmler 1989; Himmler et al. 1989). As such, tau isoforms can contain either three or four microtubule-binding domains as well as a 29 or 58 amino acid insert in the amino-terminal region (Goedert et al. 1989; Himmler 1989; Himmler et al. 1989). Tau has been found to be a flexible molecule due in part to its pro-gly motifs (Lichtenberg et al. 1988). This flexibility results in the presentation of different epitopes responsible for differential staining patterns with various monoclonal tau antibodies (Loomis et al. 1990; Brady et al. 1995; LoPresti et al. 1995). As such, confirmation of tau in the nucleolus has been controversial due to the inability of all tau antibodies except for Tau-1 to recognize nucleolar tau in situ.

In the present study, we have used sense and anti-sense tau cDNA transfection strategies to establish the presence of tau in the nucleolus of CG neuroblastoma cells. CG cells contain nucleolar tau and very small amounts of cytoplasmic tau. CGs that were transfected with tau sense expression plasmids showed an increase in both cytoplasmic and nucleolar Tau-1 staining intensity as compared to nontransfected cells. Transfection

of these cells with tau anti-sense expression plasmids abolished nucleolar Tau-1 staining. These results demonstrated that tau was present in both the nucleolus and cytoplasm, indicating that tau may also be a nucleolar protein possibly involved in ribosomal biogenesis and/or rRNA transcription in neuronal cells.

MATERIALS AND METHODS

Cell Culture

Cells were cultured on 100 mm^2 plates. CG cells (human neuroblastoma) were cultured in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Walkersville, MD) supplemented with 5% horse serum, 20 mM L-glutamine, 10 µg/ml penicillin/streptomycin, and 10 mM sodium pyruvate (BioWhittaker). All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Plasmid Construction

Tetracycline inducible sense and anti-sense constructions were made by first isolating the full length, four repeat tau cDNA (htau40; Goedert et al. 1989) from the plasmid pT7HT/htau40 cDNA (Carmel et al. 1996). The tau anti-sense cDNA was cloned by a *BamHI/NcoI* digest and blunt ligated into the *XbaI*/blunt digested pHUD 10.3 plasmid which contained the heptamerized tetO sequences (Gossen and Bujard 1992; a gift from Dr. Bujard at University of Heidelberg). To construct the tau sense plasmid, the tau anti-sense insert was *BamHI* digested and ligated into the *BamHI* site of the pHUD 10.3 vector. In conjunction with the plasmid 15-1neo, derived from pHUD 15.1 (Gossard and Bujard 1992), which encoded the tTA (tetracycline repressor VP16 activator complex), tetracycline regulated expression was established. Upon withdrawal of tetracycline, the tTA activated the pHUD10.3 vector through the tetO enhancer

sequences. Restriction analysis and dideoxy DNA sequencing, using an applied Biosystems 373 automated sequencer (Watanebe et al. 1996), confirmed the integrity of these constructs. The humanized mutant green fluorescent protein (hGFP) plasmid, S56T (a gift from Dr. J. Backer at University of Heidelberg), encoded a fluorescent marker for microinjected and transfected cells.

Transfections

Plasmid DNA, purified using Quiagen Tip-100s or by cesium chloride banding, was introduced into CG cells by the calcium phosphate method as previously described (Watanebe et al. 1996). Cells were examined 48 h posttransfection by indirect immunofluorescence.

Immunofluorescence

Cells were subsequently fixed in 3% ultrapure TEM grade formaldehyde (Tousimis Research Corp., Rockville, MD) in 100 mM TRIS-HCl, pH 7.4 (Buffer A), for 30 min at 24°C. Cells were rinsed in Buffer A for 5 min, permeabilized in 0.5% Triton-X-100 in Buffer A for 5 min, and then reduced in 0.5 μg/ml $NaBH_4$ in Buffer A for 4 min. Cells were then processed for indirect immunofluorescence microscopy using previously described methods (Zinkowski et al. 1991). The preparations of cells were rinsed in Buffer A and coverslipped with 0.1% *p*-phenylenediamine (Sigma) in a 9:1 mixture of PBS:glycerol.

Quantitation of Immunofluorescence

After the slides were processed for immunofluorescence, video images of nucleoli were captured and stored on the computer. Samples processed for immunofluorescence were analyzed using a Zeiss LSM confocal microscope (Carl Zeiss, Inc., Thornwood,

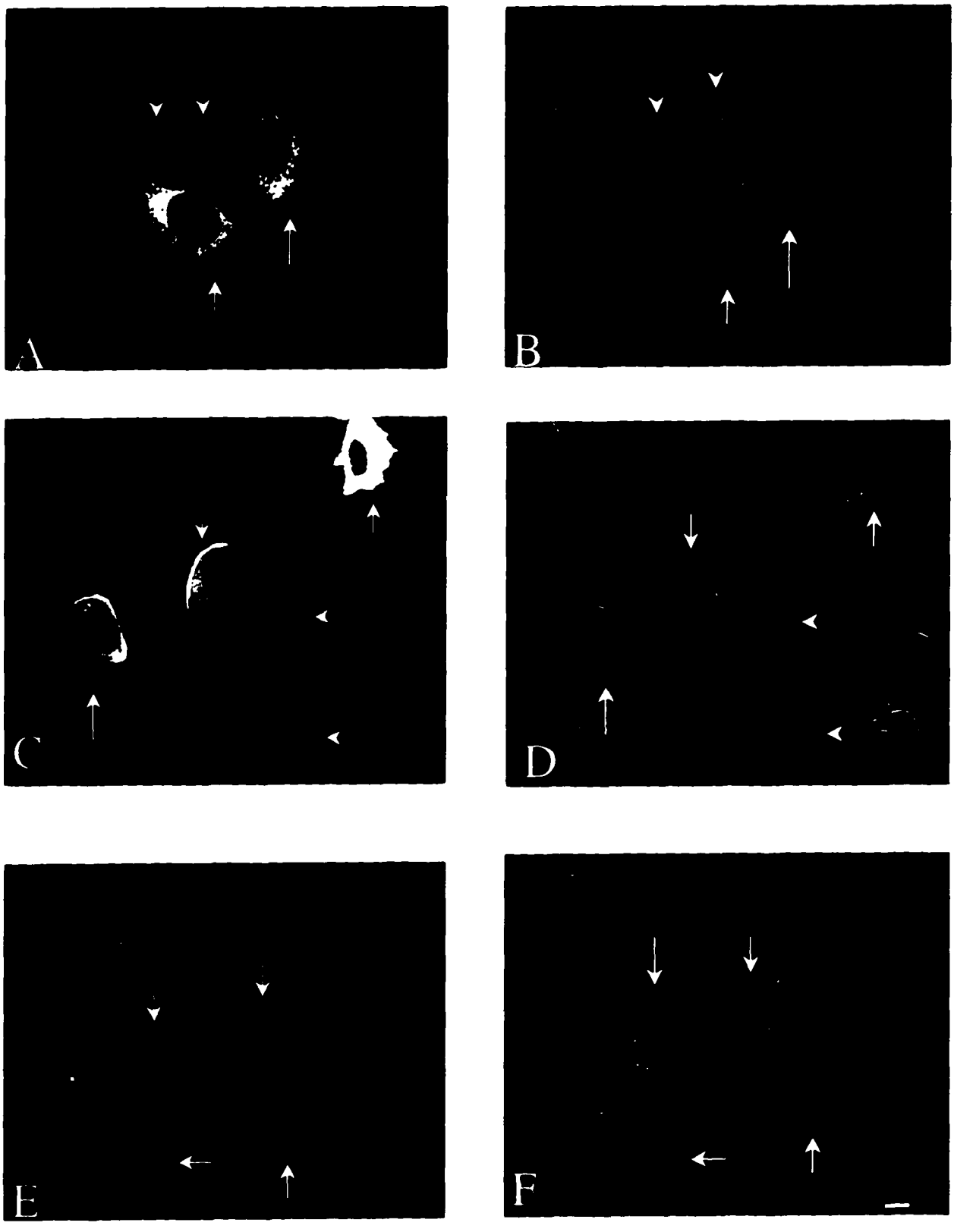
NY) coupled to the Metamorph image analysis system (Universal Imaging, Westchester, PA) and an IBM compatible PC 486/33. Image processing was performed by setting the brightness and contrast controls on the confocal microscope to provide an optimal signal. Image quantification was performed by thresholding the area to be quantitated and subsequently measuring the gray level of each pixel. Each pixel has a gray scale that ranges from 0 to 255. The pixels of each thresholded area were summed and the final measurement was recorded to the hard drive. The resultant data were imported into Excel and the fluorescence intensity of the cytoplasm and nucleus of each cell was analyzed.

RESULTS

Immunofluorescence Analysis of Tau Sense Transfected CG Cells

Recent studies demonstrated that the monoclonal antibody Tau-1 reacts with tau found in the nucleolus of the CG neuroblastoma cell line (Loomis et al. 1990; Wang et al. 1993). To determine whether this nucleolar Tau-1 signal was due to tau or a cross-reacting species, these cell lines were transfected with sense and anti-sense tau cDNA expression vectors. CG cells transfected with hTau40 cDNA and immunostained with Tau-1 showed a dramatic increase in Tau-1 staining intensity. Tau-1 staining was present in the cytoplasm in a diffuse manner throughout the cell (Fig. 1A, B; arrows) as compared to nontransfected cells which contained little to no cytoplasmic tau (Fig. 1A, B, E, F; arrowheads). Immunofluorescence of transfected cells demonstrated a variable staining pattern in the nucleolus which ranged from a strong fluorescence intensity in the nucleolus of some cells (Fig. 1C, D; arrows) to others which contained no detectable nucleolar Tau-1 staining (Fig. 1C, D; arrowheads). Nontransfected cells seen in Fig. 1C do exhibit nucleolar Tau-1 staining, but barely visible due to the dynamic range of the

Fig. 1. CG neuroblastoma cells immunostained with Tau-1. **A** CG cells transfected with htau40 cDNA expression vector. *Arrows* indicate transfected cells. *Arrowheads* indicate nontransfected cells. **B** Corresponding phase image of cells in **A**. **C** CG cells transfected with htau40 cDNA expression vector. *Arrows* indicate transfected cells lacking Tau-1 staining in the nucleolus. *Arrowheads* indicate transfected cells with Tau-1 staining in the nucleolus. **D** Corresponding phase image of cells in **C**. **E** Nontransfected CG cells. *Arrows* indicate nontransfected cells. **F** Corresponding phase image of cells in **E**. Bar represents 5 μm



camera. In cells which contained no detectable nucleolar tau, the cells appeared to be more rounded and have an abnormal nucleolar morphology as seen by phase analysis (Fig. 1D; arrows). Cells transfected with the expression vector without the tau sense cDNA insert displayed normal cellular morphology and nucleolar Tau-1 staining (data not shown). Thus the increase in Tau-1 staining intensity in transfected cells resulted from the introduction and expression of tau cDNA containing plasmids into these cells, as opposed to nonspecific effects of the transfection procedure itself.

Quantitation of Tau-1 Staining Intensity in Tau Sense Transfected CG Cells

To determine if the transfected tau was actually localizing to the nucleolus and causing an increase in Tau-1 staining, it was decided to measure the amount of Tau-1 staining in the nucleolus of transfected cells. The intensity of fluorescent staining as measured by relative light units (RLUs) of the nucleoli and cytoplasm of each cell was quantitated by image analysis, as described in Materials and methods. The total fluorescence intensity of Tau-1 staining in the cytoplasm was significantly increased in all transfected cells (100,000 to 5,000,000 RLUs) as compared to nontransfected cells (1,000 to 100,000 RLUs; Fig. 2). Nucleolar Tau-1 immunofluorescence was also significantly increased in some transfected cells and seemingly abolished in other transfected cells when compared with nontransfected cells (Fig. 3). Nucleolar staining intensities in transfected cells ranged from no staining (0-500 RLUs) in approximately 28% of the cells to RLUs of over 20,000-80,000 in 17% of transfected cells. The remaining transfected cells (approximately 65%) exhibited RLUs in the range of 500 to 20,000 as did the nontransfected cells. In some transfected cells there appeared to be a correlation between no Tau-1 staining in the nucleolus and large amounts of cytoplasmic tau. A comparison

Fig. 2. Total Tau-1 fluorescence intensity of CG neuroblastoma cytoplasm. Each *bar* represents the percentage of cells that stained at a given range of fluorescence intensity (n=100)

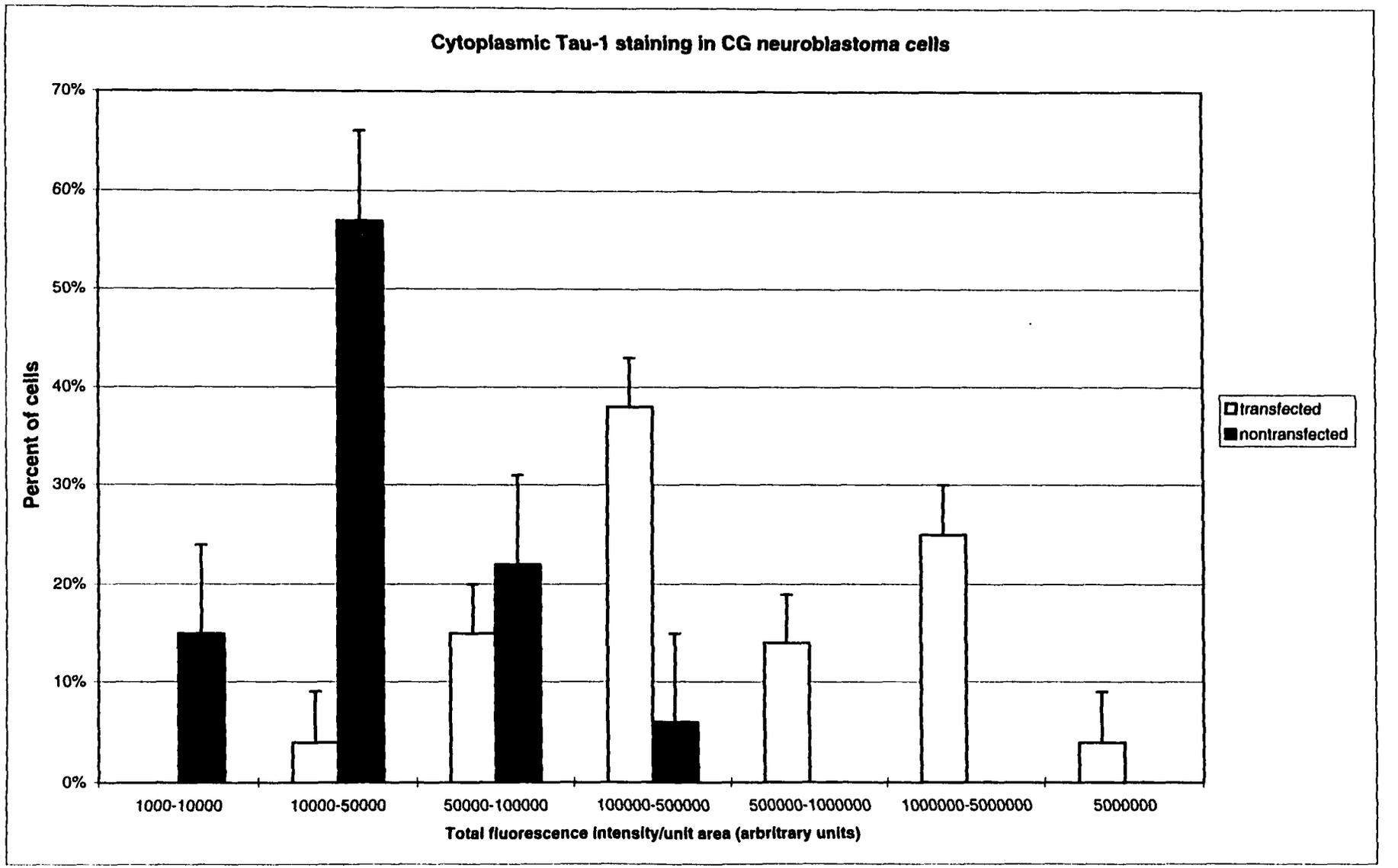
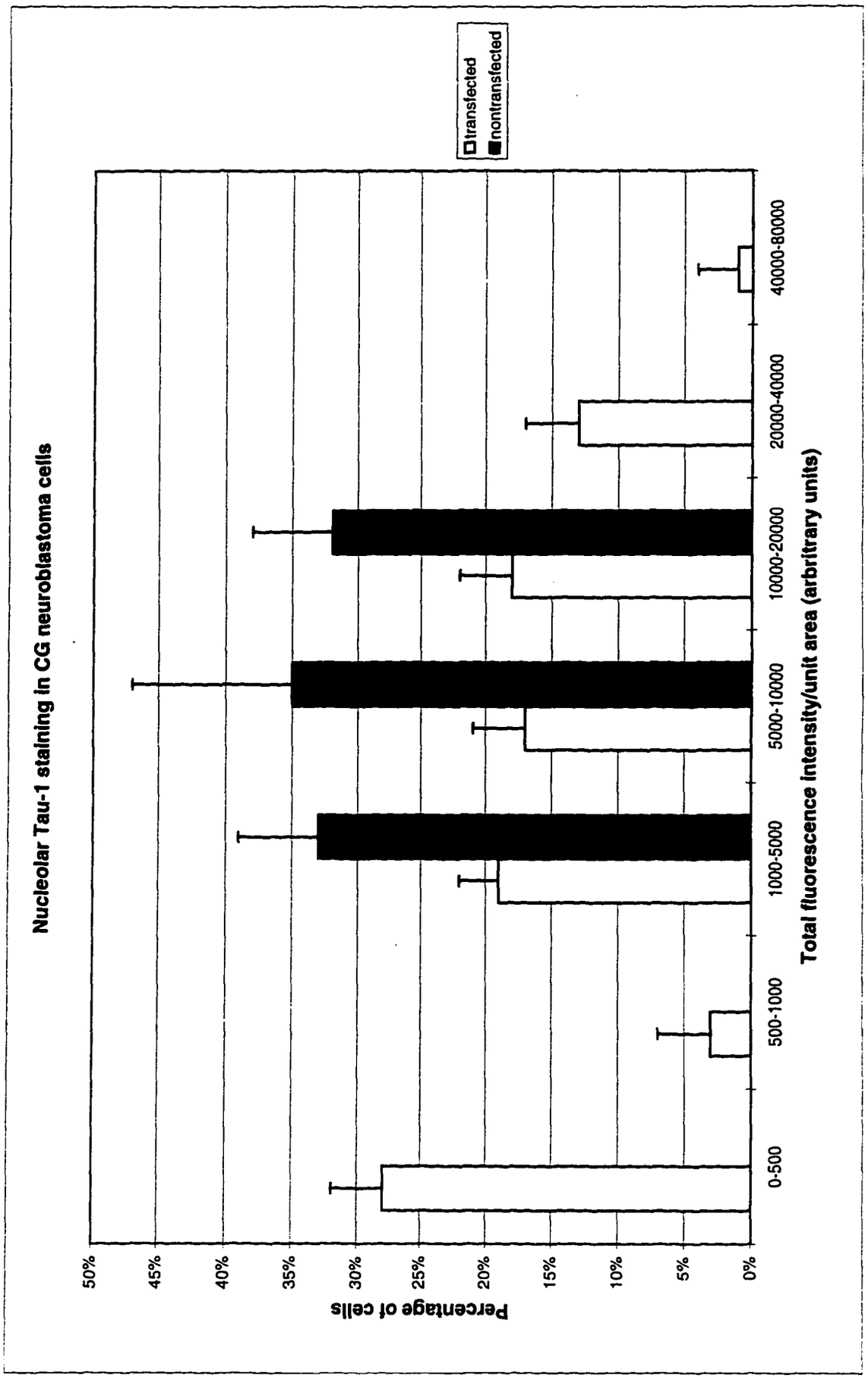


Fig. 3. Total Tau-1 fluorescence intensity of CG neuroblastoma nucleoli. Each *bar* represents the percentage of cells that stained at a given range of fluorescence intensity (n=100)



of cytoplasmic versus nucleolar Tau-1 staining in transfected CG cells (Fig. 4) established that, with the exception of one instance, cells containing markedly large Tau-1 staining intensities in the cytoplasm (>2,000,000 RLUs) tend to display decreased nucleolar Tau-1 staining (<1,000 RLUs). Note that three regions exist on the scattergram in Fig. 4: 1) low cytoplasmic and low nucleolar tau staining which falls in the range of non-transfected cells; 2) low cytoplasmic and medium nucleolar tau staining, indicating an increase in nucleolar tau in the transfected cells; and 3) high cytoplasmic and little to no nucleolar tau staining. These results suggested that the amount of tau in the nucleolus may be related negatively to the amount of tau in the cytoplasm of cells transfected with the tau sense expression plasmid (see Discussion).

Effects of Tau Sense Transfections on Endogenous Microtubules

Tau was originally discovered as a microtubule-associated protein (Cleveland et al. 1977), and was found to function in microtubule assembly and stabilization (for review, see Goedert 1993). To determine if the large amounts of cytoplasmic tau had an effect on the morphology of endogenous microtubules, CG cells were transfected with tau sense cDNA and processed for tubulin immunofluoresence. Microtubule bundling was observed at the periphery of the cells containing large amounts of cytoplasmic tau (Fig. 5A, B; arrows), while those containing significantly less cytoplasmic tau had normal microtubule arrays (Fig. 5C, D; arrows), as did nontransfected cells (Fig. 5E, F). The results were consistent with a role of forced overexpression of tau in altering microtubular functions in transfected cells (Kanai et al., 1989).

Fig. 4. Total Tau-1 fluorescence intensity of transfected CG neuroblastoma cells comparing cytoplasmic staining to nucleolar staining. Each *diamond* represents one cell (n=50)

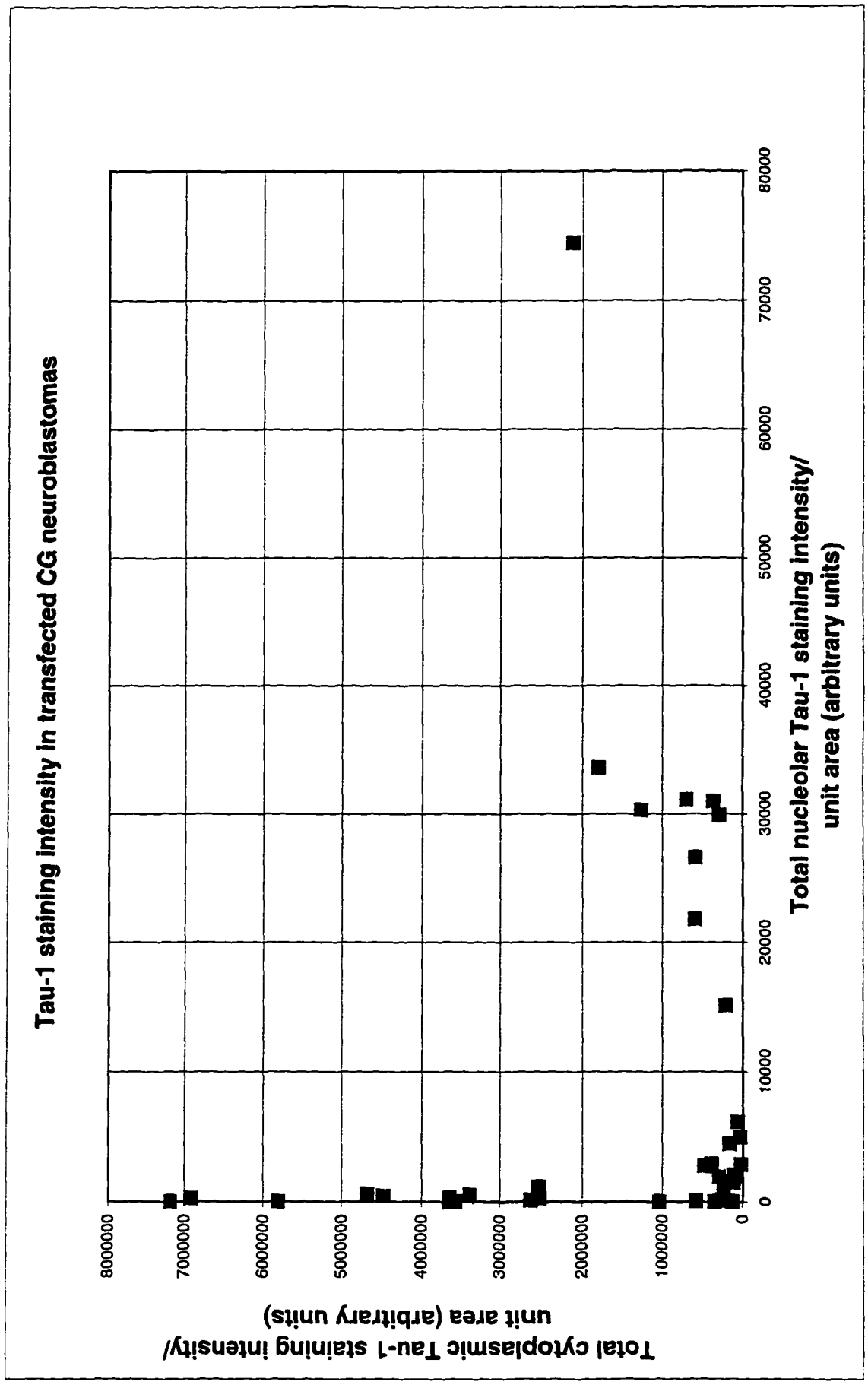
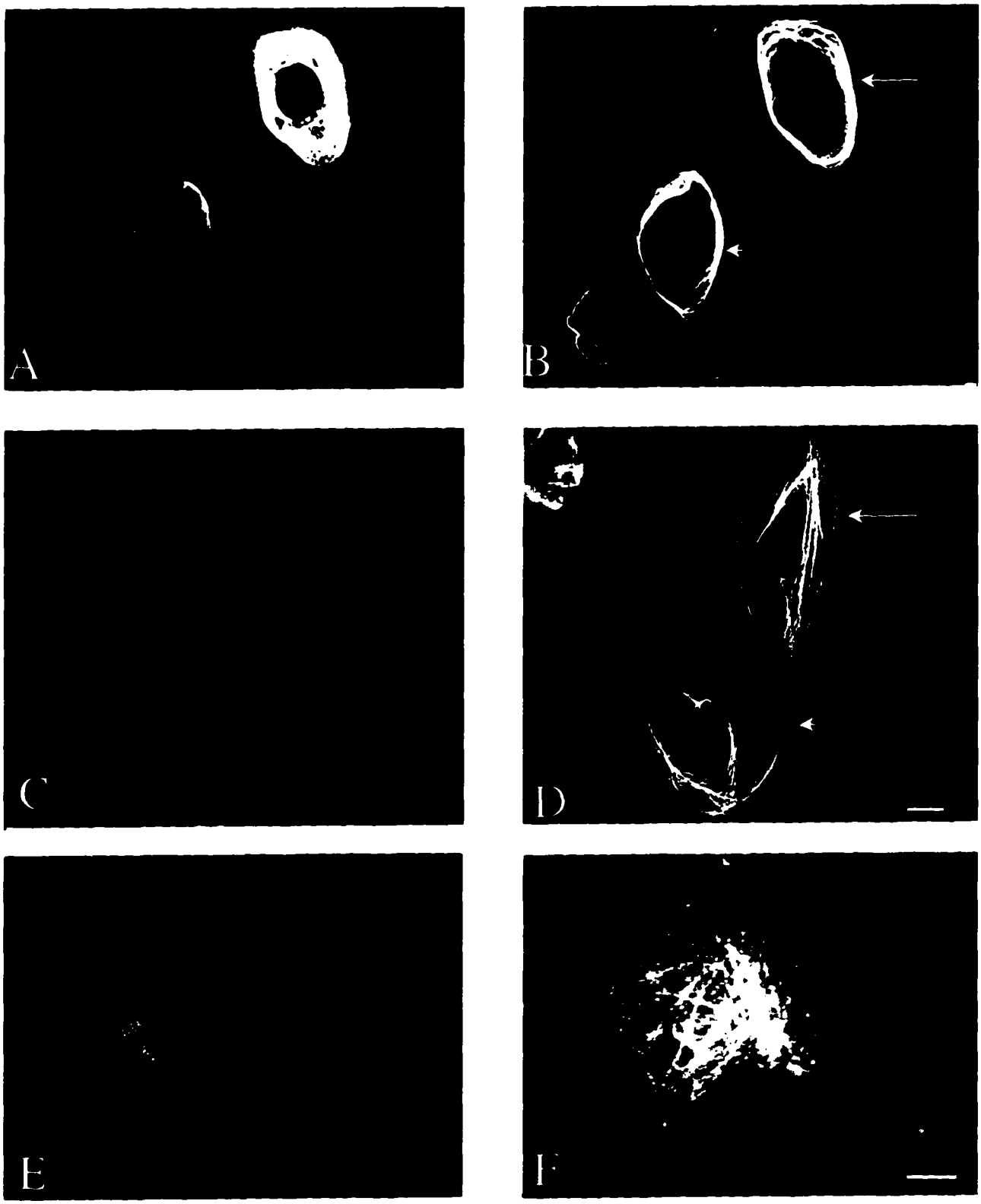


Fig. 5. CG neuroblastoma cells transfected with tau sense cDNA expression vectors. **A** CG cells transfected with tau and immunostained with Tau-1. **B** Corresponding image of cells in **A** immunostained with 5H1. *Arrows* indicate transfected cells with microtubule bundling. **C** CG cells transfected with tau and immunostained with Tau-1. **D** Corresponding image of cells in **C** immunostained with 5H1. *Arrows* indicate transfected cells with normal microtubule arrays. Bar represents 5 μm. **E** Nontransfected CG cells immunostained with Tau-1. **F** Corresponding image of cells in **E** immunostained with 5H1. Bar represents 10 μm



Immunofluorescence and Quantitative Analysis of Tau Anti-sense Transfected CG Cells

To determine whether the nucleolar Tau-1 signal was due to tau or a cross-reacting species, these cells were also transfected with anti-sense tau cDNA expression vectors. The tau anti-sense plasmid and the green flourescent protein reporter plasmid (GFP S65T) were transiently cotransfected into CG neuroblastoma cells. The use of the GFP S65T plamid enabled us to locate transfected cells easily. The anti-sense cDNA was believed to form RNA-DNA hybrids with the endogenous sense sequence, thus reducing the efficiency of translation, stability, or transport of the mRNA concerned (Eguchi et al. 1991). Compared to nontransfected cells (Fig. 6A, B; arrowheads), the cells transfected with tau anti-sense plasmid had little to no Tau-1 staining (Fig. 6A, B; arrows). Thus nucleolar staining by Tau-1 in nontransfected CG cells was essentially eliminated in cells transfected with anti-sense tau, supporting our contention that Tau-1 immunoreactivity in the nucleolus of CG cells was due to tau rather than a cross-reacting species. Additionally, cellular morphology of transfected cells does not appear to be affected, as observed by phase microscopy (Fig. 6B; arrows). Using immunofluorescence quantification, Tau-1 staining of nucleoli in CG cells transfected with tau anti-sense was compared to those not transfected. Fluorescence quantitation showed 100% staining in nontransfected cells and less than 5% staining in transfected cells (Fig. 6C). Reduction in Tau-1 levels in transfected cells relative to nontransfected cells resulted from sufficient anti-sense tau RNA to diminish tau synthesis directed by mRNA from the endogenous gene.

Fig. 6. CG neuroblastoma cells transfected with the tau anti-sense cDNA expression vector. **A** CG cells transfected with tau anti-sense and immunostained with Tau-1. **B** Corresponding phase image of cells in **A**. *Arrows* indicate transfected cells. *Arrowheads* indicate nontransfected cells. Bar represents 5 μm. **C** Percentage of Tau-1 immunostaining nucleoli in tau anti-sense transfected and nontransfected CG neuroblastomas. *Bars* represent percent of nucleoli immunostained with Tau-1 (n=50)

DISCUSSION

Due to the fact that the Tau-1 monoclonal antibody is the only tau antibody to date to react with nucleoli and NORs by immunofluorescence, there is still some controversy over tau's presence in this organelle. Previously, our laboratory demonstrated the presence of what appeared to be several isoforms of tau in isolated primate nuclei subjected to immunoblot analyses. This was especially notable using CG neuroblastoma cells, which were shown to contain little to no discernible levels of cytoplasmic tau but tested positive for nuclear tau with antibodies whose epitopes nearly spanned the tau molecule (Loomis et al. 1990).

Except in diseased states (Papasozomenos 1995), localization of tau to the nucleus or nucleolus of human brain has not been reported. It was not until nuclei purified from frozen brain were processed using the same fixation protocols as cells in culture that tau localization to the nucleus was observed (Brady et al. 1995). Our laboratory reported the presence of tau in isolated brain nuclei using three monoclonal antibodies (Brady et al. 1995) in which immunofluorescent staining was observed to be mainly punctate and extranucleolar, although some nucleolar staining was observed. However, these differences in immunolocalization may be due to differences in cell cycle and/or cell differentiation. The fact that few of the nuclei exhibited nucleolar tau staining suggested that perhaps tau's nucleolar function in terminally differentiated cells of neurons was intermittent or no longer necessary.

Additionally, fixation and sequence epitope specificity of the monoclonal tau antibody used was also shown to affect the localization of tau in oligodendrocytes (LoPresti et al. 1995). Only the monoclonal antibody Tau-5 localized to oligo-

dendrocytes in situ, whereas a number of other tau monoclonal antibodies stained cultured oligodendrocytes. Tau-5 immunostained the cytoplasm in the cell body of rat oligodendrocytes but not axons under the fixation conditions employed. We have also discovered that overfixation of cultured cells abolished the nuclear tau signal in immunohistochemical experiments, while formic acid treatment greatly increased the intensity of the nuclear tau signal (P. A. Loomis and L. I. Binder unpublished observations). Possibly the destruction or masking of tau epitopes by traditional in situ fixation techniques caused the lack of immunofluorescent staining in sections from human brain, as is appears to do in oligodendrocytes.

Papasozomenos (1995) localized tau within neuronal nuclei from an autopsy case of presenile dementia with motor neuron disease. Immunofluorescence was observed to be diffuse throughout the nucleus, but excluding the nucleolus. He hypothesized that this localization of tau in the nucleus was in response to chronic stress underlying the pathology of the disease and that tau may play a role in the stress response, acting similarly to heat shock proteins. Heat shock proteins were known to migrate transiently into the nucleolus, redistribute in the nucleus, and exit into the cytoplasm following heat shock (Welch and Mizzen 1988); these proteins may also be required for the transport of certain proteins into the nucleus (Shi and Thomas 1991).

Other characterizations of tau in the nucleus include experiments carried out by Lu and Wood (1993), who found that bovine tau microinjected into CHO cells localized both to the centrosome and to the nucleolus. Five to ten minutes after injection, staining of the centrosome decreased with an accompanying increase in tau on the microtubule network. Nucleolar staining remained unaffected throughout the entire experiment,

indicating that tau was directionally targeted to the nucleolus, where it functioned in a different capacity than cytoplasmic tau. Tau has also been localized to the nucleus in a neuritogenic rat CNS cell line that was immunostained with a tau polyclonal antibody and PHF-1, an antibody recognizing tau phosphorylated at Ser 396 and 404 (Lambert et al. 1995). Several days after plating, two populations of cells could be observed, those which had both nuclear and cytoplasmic PHF-1 labeling and those which had only cytoplasmic labeling. These cells were also transfected with tau sense and anti-sense oligonucleotides, and the tau anti-sense transfection decreased both nuclear and cytoplasmic PHF-1 immunofluorescence. No mention was made of the effect of tau sense on nuclear PHF-1 immunofluorescence, and no nucleolar localization of tau was reported. Taken together, these observations suggested that tau can be found both in the nucleolus and throughout the nucleus; the reasons for this differential location have yet to be determined.

Our experiments utilizing quantitation by image analysis demonstrated a significant increase in both nucleolar and cytoplasmic tau in cells transfected with tau sense expression plasmids. Because the cells were transiently transfected with the tau cDNA plasmid, the level of expression varies from cell to cell, depending on the number of gene copies present. In looking at many cells, we found that not only did the intensity of the cytoplasmic staining vary with the level of expression, cells expressing high levels of cytoplasmic tau actually exhibited little to no nucleolar staining. Previous tau transfection studies (Lee and Rook 1992; Haque et al. 1995) showed that the intensity of antibody staining varied from one cell to another in both transient and stable transfectants and was independent of the number of passages in stably transfected cells. In our study, a

comparison of cytoplasmic versus nucleolar Tau-1 staining in transfected CG cells established that cells containing markedly large Tau-1 staining intensities in the cytoplasm have decreased nucleolar Tau-1 staining. These results suggested that the amount of tau in the nucleolus may be regulated negatively to the amount of tau in the cytoplasm of transfected cells. The lack of nucleolar tau in cells containing large amounts of cytoplasmic tau may be due to congestion in the cytoplasm inhibiting translocation into the nucleus, possibly in conjunction with the bundling of microtubules in the cytoplasm seen in some transfected cells. However, interpretation of these results was clouded by the fact that some cells with relatively modest amounts of cytoplasmic tau also showed a loss of nucleolar signal.

Microtubule bundling was observed at the periphery of cells containing large amounts of cytoplasmic tau and little to no nucleolar tau, while those containing smaller amounts of cytoplasmic tau had normal microtubule arrays. Lee and Rook (1992) also detected microtubule bundling in cells transfected with four-repeat tau cDNA. Bundles were seen as sharp spikes emanating from the cell or as thick dense bands running along the edge of the cell. An average of 20% of transfected cells containing bundled microtubules, which is consistent with our results. They also determined that bundled microtubules were seen mainly in cells with high levels of tau expression. Barlow et al. (1994) observed that the degree of bundling in CHO cells transfected with full length tau cDNA also appeared to correlate with the level of tau expression. Cells with weak tau fluorescence exhibited little evidence of microtubule bundling. Many of the cells with the highest tau expression had circular bundles of microtubules around the nucleus or periphery of the cell. As mentioned above, cells expressing large amounts of Tau-1

staining in the cytoplasm exhibited microtubule bundling at the periphery of the cell or circling the nucleus. These cells also had an abnormal cytoplasmic and nuclear morphology. Again, such results are consistent with a role of forced overexpression of tau in altering microtubule functions in transfected cells that most likely may lead to a disruption in cellular transport which, in this case, may affect the import of tau into the nucleus.

To determine if a phosphorylation event at the Tau-1 epitope caused the lack of Tau-1 staining in the nucleolus of some cells, CG cells transfected with tau sense cDNA were treated with alkaline phosphatase prior to immunofluorescence (data not shown). Cells that were treated with alkaline phosphatase contained the same variability in nucleolar staining as untreated cells. A battery of tau antibodies were also tested which spanned the length of the molecule in case the Tau-1 epitope had undergone a conformational change and was not accessible to the Tau-1 antibody. All antibodies tested detected increased cytoplasmic tau but were unable to detect nucleolar tau in the transfected cells (data not shown). This is consistent with earlier work from our laboratory (Loomis et al. 1990) documenting that the localization of tau to the nucleolus can only be accomplished using Tau-1.

Most of the transfected cytoplasmic tau did not appear to be associated with microtubules in the CG neuroblastomas. This result had also been documented in an undifferentiated murine N_2A neuroblastoma cell line (Wang et al. 1993) and was consistent with the report of fetal tau transfected PC12 cells in which the expressed tau appeared to be mainly cytoplasmic and not associated with microtubules (Leger et al. 1994). Esmaeli-Azad et al. (1994) also found that PC12 cells transfected with tau sense

plasmids produced tau that was not associated with the cytoskeleton, although NGF treatment led to a marked increase in the fraction of cytoskeletally associated tau. Other immunocytochemical studies indicated that in transfected CHO cells, tau was expressed both unbound and bound to microtubules (Haque et al., 1995). All of these reports underscored the point that tau likely had non-microtubule-related functions, and it will be instructive to learn whether these functions were at all related to the functions(s) of tau in the nucleus.

Transfection of CGs with tau anti-sense showed almost a complete absence of nucleolar tau. These experiments codify tau as a nucleolar protein of unknown function. Confounding this finding further was the observation that nucleolar morphology was unaffected by tau's absence. However, it should be noted that the tau anti-sense expressing cells were not stably transfected; hence, any subtle affects were not given the opportunity to "breed true" through numerous cell cycles.

In summary, by using sense and anti-sense transfection techniques it was possible to establish that nucleolar Tau-1 immunostaining in the CG neuroblastoma cell line was due to the presence of tau in the nucleolus and not a cross-reacting species. Tau's specific localization to the nucleolus in these cells suggested that it had a functional role in biosynthetic pathways associated with ribosome synthesis and perhaps RNA processing. Since tau localized to the sites associated with these functions, it was hypothesized that cellular tau was more than a MAP, perhaps functioning as an important protein in ribosomal biogenesis or protein biosynthesis.

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CONCLUSION AND PROSPECTUS

In this study we have described discoveries of the localization of tau outside the central nervous system (CNS), upregulation of tau in actively dividing cells, and confirmation of tau in the nucleolus. In the first section of this dissertation, staining of interphase nucleoli and mitotic NORs in HeLa cells, nontransformed fibroblasts, and whole blood lymphocytes using the Tau-1 monoclonal antibody was reported. Tau-1 staining was detected in the nucleolus of interphase lymphocytes and on the NORs of mitotic chromosomes. This localization of tau outside the CNS and in the nucleus was unusual in that tau was originally thought to be strictly a neuronal protein and to be only associated with microtubules. Papasozomenos and Binder (1987) were the first to localize tau to ribosomes and demonstrated a localization of tau not associated with microtubules. The presence of tau in the nucleus had also been reported in nuclei isolated from frozen human brain using three monoclonal antibodies (Brady et al. 1995). Lu and Wood (1993) demonstrated that when fluorescently labeled bovine tau was microinjected into Chinese hamster ovary cells (CHO), which contained no tau mRNA or tau protein (Wang et al. 1993), the tagged protein localized both to the centrosome and to the nucleolus. Greenwood and Johnson (1995) also confirmed the presence of tau in the nucleolus by indirect immunofluorescence using LA-N-5 neuroblastoma cells. Future studies are needed to determine if tau is located in other cell types, as well as in nonprimate cells, and what role tau plays in the nucleus.

Interestingly, Tau-1 staining in the cell types studied varied from one NOR to the next but remained the same in each chromosome spread, reminiscent of silver-staining patterns of NOR proteins. Silver staining was a heritable characteristic in that the silver deposition on specific NORs was not only consistent within an individual but was also inherited (Markovic et al. 1978). Our results suggest that the Tau-1 staining intensity of NORs on specific acrocentric chromosomes may be a heritable trait similar to silver staining, although this remains to be tested in definitive family studies. However, Tau-1 antibody staining does not appear to correlate with silver staining of the NORs and is presumably indicative of some other functional state of the NOR. Hence future research directions may involve determining the heritability of Tau-1 staining as a marker for a yet to be determined NOR function that lends itself to cytogenetic analysis.

Preliminary evidence has also suggested that transformed lymphocytes stained more intensely with Tau-1 than those freshly isolated from blood. Quantification of staining intensity showed an increase in total Tau-1 immunofluorescence in PHA-stimulated cells. Upregulation of total nuclear tau would be expected in cells stimulated to divide and in need of an increased rRNA output prior to division if tau were involved in some aspect of rRNA synthesis, processing, or transport. Cell fractionation of pulse labeled cells might lend insight as to what other nucleolar proteins are upregulated following PHA stimulation. Experiments to determine which of these were tau-binding proteins could be readily performed, followed by protein identification. Another avenue of interest would be to determine what effect differentiation of cultured neuroblastoma cells would have on nucleolar tau, i.e., would it be downregulated since the cell is no longer actively dividing?

Since nuclear tau also has properties similar to PHF tau in AD, the staining intensity of Tau-1 in lymphocytes of AD patients was studied versus age-matched controls. In the second section of this dissertation, quantitated staining intensity showed that there was no significant difference in total Tau-1 immunofluorescence in nonstimulated lymphocytes between AD or age-matched controls, as well as no significant difference in staining intensity in PHA-stimulated lymphocytes between AD and age-matched controls. We did not have an opportunity to quantitate the fluorescence intensity between nonstimulated and PHA-stimulated lymphocytes in Alzheimer's patients, but that would be the next step in determining if nucleolar Tau-1 staining was affected in Alzheimer's Disease, which might lead to a noninvasive diagnostic marker for Alzheimer's Disease.

In the third section of this dissertation, our studies confirm the presence of tau in the nucleolus of CG neuroblastoma cells by the use of tau sense and tau anti-sense transfection strategies. Our results suggested that the amount of tau in the nucleolus may be regulated negatively to the amount of tau in the cytoplasm of transfected cells. The lack of nucleolar tau in cells containing large amounts of cytoplasmic tau may be due to congestion in the cytoplasm inhibiting translocation into the nucleus, possibly in conjunction with the bundling of microtubules in the cytoplasm seen in some transfected cells. Microinjection of different protein concentrations of tau into these cells might determine if large amounts of cytoplasmic tau were related to a decrease in nucleolar tau or bundling of microtubles. The lack of nucleolar tau may also be related to which isoform of tau is transfected; possibly only certain isoforms could be found in the

nucleolus, and microinjection of the various isoforms of tau may determine if this is the case.

The nucleolar Tau-1 signal was determined to be due to tau and not to a cross-reacting species, since transfection of CGs with tau anti-sense showed an almost complete absence of nucleolar tau. Tau's specific localization to the nucleolus in these cells suggested that it had a functional role in the nucleus. Due to the problems in isolating nucleolar tau, little is known of its role in the nucleus. To determine what role nucleolar tau plays in the nucleolus, future studies might include the treatment of neuroblastoma cell lines with actinomycin D (AMD) which affects ribosome formation and nucleolar structure (Hadjiolov 1985). In veterbrate cells, AMD has been found to be a strong and selective inhibitor of ribosomal RNA synthesis (Perry and Kelley 1970) and has resulted in a characteristic reorganization of the ultrastructural components of the nucleolus (Simard et al. 1974). After this reorganization, three areas are recognized in the segregated nucleolus, each one corresponding to a coalescence of the three major ultrastructural components of the nucleolus, the fibrillar center, the dense fibrillar component, and the granular component. AMD treatment in conjunction with immunofluorescence could improve our understanding of the localization of tau in the nucleolus and therefore suggest possible functions, as well.

If nucleolar tau was involved in rDNA transcription, treatment of these cells with the drug 5,6-dichloro-1-B-D-ribofuranosylbenzimidazole (DRB) would induce reversible unfolding of the interphase nucleolus into extended beaded strands without inhibiting transcription of the rRNA genes (Scheer et al. 1984). Each of the beads was thought to contain a single rDNA transcription unit (Scheer et al. 1984; Haaf et al. 1991). When

probed with Tau-1, the nucleolar "necklaces" might appear as a linear array of fluorescent entities, indicating that tau was involved in some aspect of rDNA transcription.

Molecular genetic studies of nucleolar tau would be advantageous in determining which isoforms of tau were located in the nucleolus, as well as in making cDNA deletion mutants to determine if there was a nuclear localization signal (NLS). Tau does not appear to contain a canonical NLS and may be too large to diffuse rapidly into the nucleus, but it may have a novel NLS that has yet to be identified (S. Adams, personal communication, March 21, 1997). The microtubule-binding domain of tau has been found to be highly basic and may actually be the NLS which consists of a stretch of 3-5 basic residues, often flanking a proline or glycine residue (Chelsky et al. 1989).

In summary, tau has been found to be no longer confined to just the central nervous system or the testis (Ashman et al. 1992), but may now be found in human lymphocytes and fibroblasts as well. These studies have contributed to the growing evidence that tau was a multifunctional protein found associated not only with microtubules in the axons and somatodendritic compartments of neurons, but also with the nucleolus in neuroblastoma cells, HeLa cells, human skin fibroblasts, and lymphocytes. Nucleolar tau has also been found to be upregulated in PHA-stimulated lymphocytes; since nucleolar tau localizes to the site of ribosomal synthesis and has been found to be associated with ribosomes (Papasozomenos and Binder 1987), it can be hypothesized that nucleolar tau may be involved in ribosomal biogenesis or protein biosynthesis. However, elucidation of the definitive function of nucleolar tau has yet to be established.

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