Expression of Multiple Tau Isoforms and Microtubule Bundle Formation in Fibroblasts Transfected with a Single Tau cDNA

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Abstract. Tau proteins are a class of low molecular mass microtubule-associated proteins that are specifically expressed in the nervous system. A cDNA clone of adult rat tau was isolated and sequenced. To analyze functions of tau proteins in vivo, we carried out transfection experiments. A fibroblast cell line, which was transfected with the cDNA, expressed three bands of tau, while six bands were expressed in rat brain. After dephosphorylation, one of the three bands disappeared, demonstrating directly that phosphorylation was involved in the multiplicity of tau. Morphologically, we observed a thick bundle formation of microtubules in the transiently and stably tau-gene-transfected cells. In addition, we found that the production of tubulin was prominently enhanced in the stably transfected cells. Thus, we suppose that tau proteins promote polymerization of tubulin, form bundles of microtubules in vivo, and play important roles in growing and maintaining nerve cell processes.

MICROTUBULE is one of the main cytoskeletal elements in all eukaryotic cells and is particularly abundant in neuronal cells. It has been well known that there are several kinds of proteins that copurify with brain tubulin during repetitive cycles of temperature-dependent assembly and disassembly. Among these proteins of neuronal tissues, high molecular mass microtubule-associated proteins (MAPs; MAP1 and MAP2)[1] and tau proteins are the major species, and recently several minor proteins have also been identified (Cleveland et al., 1977*a*,*b*; Murphy and Borisy, 1975; Olmsted, 1986; Sloboda et al., 1975; Weingarten et al., 1975).

The high molecular mass MAPs, MAP1 and MAP2, form armlike projections on microtubules and assume flexible rodlike structures ~100–200 nm in length (Kim et al., 1979; Zingsheim et al., 1979; Voter et al., 1982; Vallee et al., 1983; Shiomura and Hirokawa, 1986*a*; Sato-Yoshitake et al., 1989). Recent structural studies demonstrated that the microtubule-rich domain in the nerve cells consists of microtubules and associated cross-bridges as main structures (Hirokawa et al., 1985; Shiomura and Hirokawa, 1987*a*,*b*). High molecular mass MAPs (MAP1, MAP2, 270-kD MAP) were identified as components of these cross-bridges associated with microtubules in vivo (Hirokawa et al., 1985; Hirokawa, 1986; Shiomura and Hirokawa, 1987*a*,*b*; Hirokawa et al., 1988*b*; Sato-Yoshitake et al., 1989).

Tau factors are a number of proteins in the molecular mass range of 55,000–62,000, which are shown to be closely related by both peptide sequences and amino acid compositions (Cleveland et al., 1977*a*). These proteins are specifically expressed in neuronal cells (Binder et al., 1985), and, furthermore, the highly phosphorylated tau is a major component of the paired helical filaments in Alzheimer's disease (Wischik et al., 1985; Ihara et al., 1986; Grundke-Iqbal et al., 1986*a*,*b*; Kosik et al., 1986; Wood et al., 1986). Tau promotes the polymerization of tubulin and is heat stable (Weingarten et al., 1975), and able to bind to calmodulin in the presence of calcium (Sobue et al., 1981; Lee and Wolff, 1984). Recently, we have shown that tau is a short rodlike molecule and forms shorter cross-bridges between microtubules in vitro (Hirokawa et al., 1988*a*).

In vitro studies have revealed many interesting properties of tau. However, our present knowledge of tau still has a number of major questions unsettled, in particular about its functions in vivo. First, how does tau influence microtubule networks, if expressed in cells that do not normally express tau, such as fibroblasts? Next, why does tau appear as many bands on SDS-PAGE; e.g., by the difference of mRNA and/or by the posttranslational modifications?

To investigate these questions, we used rat tau cDNA. First, we cloned and sequenced a rat tau cDNA. We then

1. *Abbreviation used in this paper*: MAP, microtubule-associated protein.

constructed the cDNA to a β-actin enhancer-driven plasmid and transfected the plasmid into a fibroblast cell line, L-cell, which does not express tau by nature. Using immunoblot analysis, we monitored the expression of the transfected gene products in these cells. Interestingly, in these transfected cells, we found by immunofluorescence and electron microscopies that the normal loose network of microtubules was considerably changed and thick bundles of microtubules were formed.

Materials and Methods

Synthesis of DNA Probe

Crude tau prepared from rat brain microtubules by the method of Herzog and Weber (1978) was further purified by reversed-phase HPLC. The purified tau was subjected to digestion with lysylendopeptidase in the presence of 5 M urea. The resultant peptides were separated by reversed-phase HPLC and their sequences were analyzed (1:SGERSGYSSPGSPGTPGSRS, 2:SRLQTAP, 3:GSLGNIHHKPGGGQV, 4:LDFK, 5:IGSLDNITHVPGGGNK, 6:LTFRENAK, 7:TDHGAEIVY, 8:SPVVSGDTSPRHLSNV, 9:VDSPQLATLA). Of several determined sequences, IGSLDNITHVPGGGNK was selected for the probe. Next, the optimized DNA sequence, "ATCGGCTCCCTGGACAACATCACCCACGTGCCCGGCGGCGGCAACAAG," was deduced from the amino acid sequence (Lathe, 1985). Then we designed 31 mer DNA " ATCGGCTCCCTGGACAACATCACCCACGTGC" from the 5′ end and complementary 28 mer DNA "CTTGTTGCCGCCGCCGGGCACGTGGGTG" from the 3′ end. Both DNAs were hybridized to each other and elongated with dATP, dGTP, dTTP, and α-^{32}P-dCTP by DNA polymerase I. The DNA synthesis was performed using DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The location of the peptide sequences in the whole molecule are underlined with their head numbers in Fig. 1 *A*.

DNA Sequencing

DNA fragments were subcloned into bluescript M13 SK and KS with the

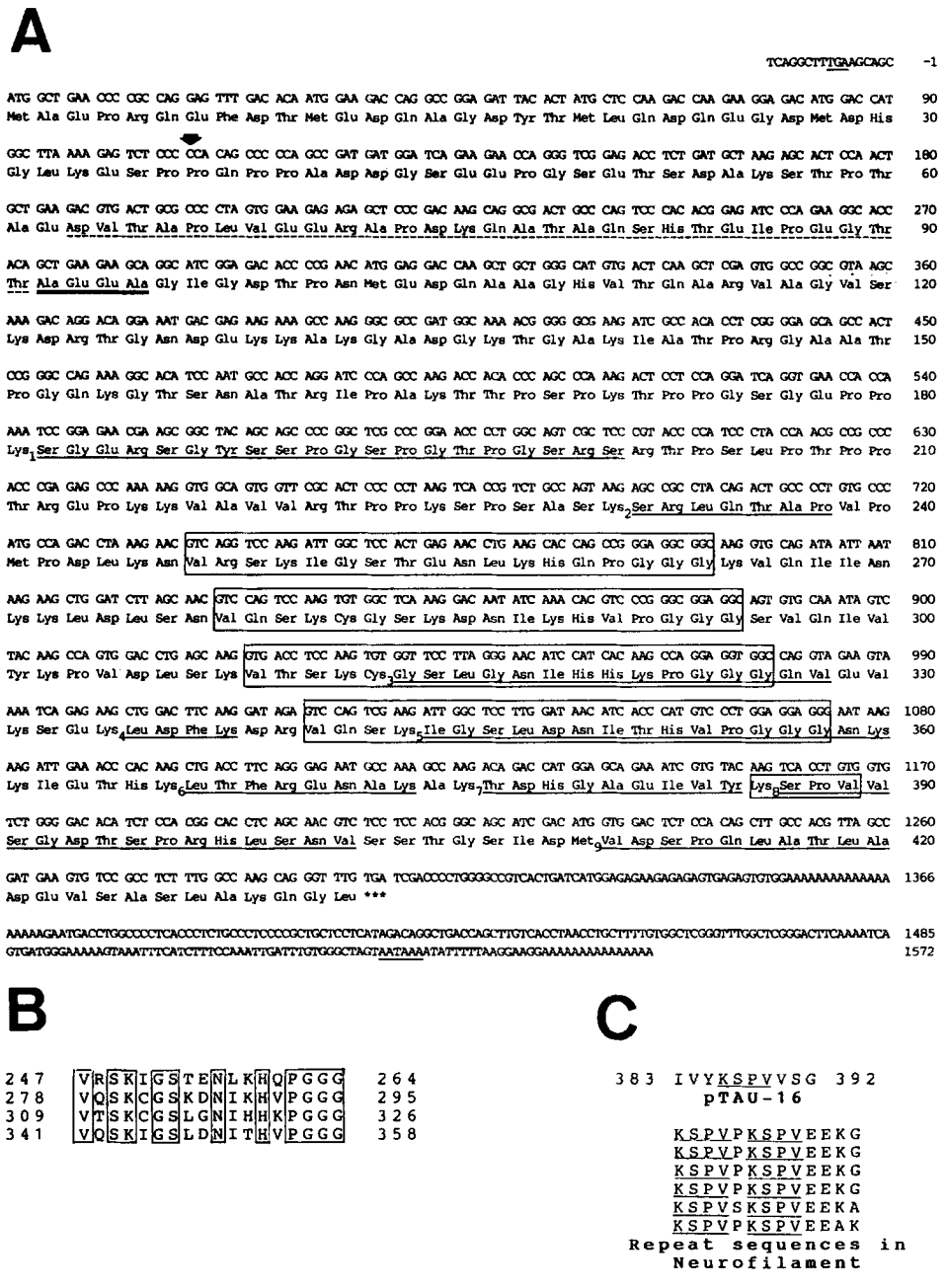

Figure 1. Primary structure of rat brain tau. (*A*) Complete sequence of pTAU-16 is shown. The predicted amino acid sequences that correspond to the sequences of the tryptic peptides are underlined with their head numbers. There are four internal repeats (*boxed area*) in the COOH-terminal domain. "Lys-Ser-Pro-Val" (*boxed area*) is the interesting repeating sequence found in neurofilament. Another clone, pTAU-15, lacked 87 bp (29aa) (- - - -) upstream the "Ala(92)-Glu-Glu-Ala" (═══) sequence. pTAU-15 included the sequence downstream of the position (↓), not reached the 5′ end. The polyadenylation signal sequence, 22 bases upstream of the poly(A) tail, is underlined. (*B*) Four internal repeat sequences are shown. Among the repeat units, the amino acids were highly conserved, and 11 out of the 18 residues were completely identical. (*C*) The repeated sequences of neurofilament-M (Myers et al., 1987) and the surrounding sequence of Ser-387 are shown respectively. "Lys-Ser-Pro-Val" is found 12 times in the 6 tandem copies of 13 residues repeat unit of neurofilament-M.

Escherichia coli strain XL-1 as host, and sequenced by the dideoxy chain termination method of Sanger et al. (1977) with overlapping restriction fragments. The cDNA was sequenced on both strands with at least two gel readings covering any particular point. We determined the sequence using Sequenase (United States Biochemical Corp., Cleveland, OH). Both DNA and deduced amino acid sequences were analyzed by using "GENETYX" (Software Development Co., Tokyo, Japan).

Transfection and G418 Selection

pTAU-16 cloned in bluescript M13 KS(+) was digested by XbaI, made blunt ended by klenow fragment and cleaved at the Hind III site. Both restriction sites existed in the polylinker sites of the vector. To construct pβactTAU16, the CAT gene (Hind III–Hpa I fragment) of pβactCAT9 was replaced with the Hind III-Xba I fragment of pTAU-16. The standard calcium phosphate technique (Xie, 1984) was used to transfect pβactTAU16 to L-cells. A 0.5 ml calcium phosphate precipitate suspension containing 1 μg pβactTAU16 and 0.1 μg pSTneo (Katoh et al., 1987) was added to a 3.5-cm dish inoculated with 10^4 L-cells the day before transfection. After 19 h, the culture was fed fresh medium, and after 16 h was prepared for observation by light and EM, or transferred to 9-cm dish with G418 at 400 μg/ml for stable selection. After 3 wk, some colonies were formed and five of them were isolated for further experiments.

SDS-PAGE and Immunoblot

Transfected or control L-cells were homogenized in buffer A (100 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM PMSF, and 10 μg/ml leupeptin). The homogenate was centrifuged at 100,000 *g* for 90 min at 2°C. The resulting crude extract was brought to 1 mM with GTP and to 20 μM with taxol. The extract was incubated at 37°C for 30 min and was centrifuged at 100,000 *g* for 30 min. The pellets were dissolved in buffer A.

SDS-PAGE of the pellets and supernatants was carried out on 7.5% gel according to the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue, scanned, and the areas of peaks were measured by densitometry (model CS9000; Shimadzu Corp., Kyoto, Japan). For the immunoblot procedure, the bands were electrophoretically transferred to nitrocellulose paper.

Dephosphorylation of Tau

We modified the dephosphorylation method of Lindwall and Cole (1984). Taxol-purified microtubule preparations in buffer A were mixed 1:1 with 0.1 M Tris buffer, pH 8.2, containing *E. coli* alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) at a concentration of at least 10 U/ml; 0.02% Na azide was included to inhibit bacterial growth. The mixture was incubated for 18–24 h at 37°C.

Immunofluorescence

Transfected L-cells were washed in a stabilizing buffer (80 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, 30% glycerol, 1 mM GTP, pH 6.6), and then permeabilized with 0.5% Triton X-100 in the stabilizing buffer at 35°C for 2 min.

The cells were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in the stabilizing buffer for 15 min, treated with 1 mg/ml $NaBH_4$ in PBS for 5 min to quench the glutaraldehyde, and then placed in an antibody buffer (PBS containing 1% BSA) for 30 min.

For double-label immunofluorescence, the cells were incubated successively with the following antibodies: mouse monoclonal anti-tau, rhodamine-conjugated goat anti-mouse IgG, affinity-purified rabbit anti-tubulin IgG, fluorescein-conjugated goat anti-rabbit IgG. All antibodies except anti-tau were diluted 1:100 in an antibody buffer. Anti-tau antibody, TAU1, a generous gift of L. I. Binder (University of Alabama, Birmingham) (Binder et al., 1985) was diluted 1:20 in the same buffer. Rhodamine- or fluorescein-conjugated second antibodies were from Cappel Laboratories Inc. (Malvern, PA). In some cases, the staining process with tubulin antibody was omitted. The cells were examined with a standard 14 epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

Electron Microscopy

Transiently transfected L-cells cultured on cover glasses were washed with PBS and then permeabilized with 0.2% Triton X-100 in the stabilizing buffer at 35°C for 2 min.

The cells were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in the stabilizing buffer for 15 min, postfixed with 0.5% OsO_4 at 4°C for 30 min, blockstained with 1% uranyl acetate, dehydrated with an increasing concentration of ethanol, and embedded in Epon 812.

Ultrathin sections were cut by an ultramicrotome (LKB Instruments, Inc., Gaithersburg, MD), stained with uranyl acetate and lead citrate and observed with an electron microscope (model No. 2,000 EX; JEOL Tokyo, Japan) at 100 kV.

Results

Isolation of cDNA Clones

First, we determined nine partial peptide sequences of rat brain tau. Then we chose one of them based on the minimal redundancy of codon sequences, and designed an oligonucleotide probe (Lathe, 1985). Approximately 640,000 recombinants of a λgt10 adult rat brain cDNA library were screened with the synthesized probe, and eleven positive plaques were identified. We sequenced them and found that some of them included all or some of the nine peptide sequences. The longest cDNA was pTAU-10 (1,490 bp), and the others were completely included within pTAU-10. However, the 5′ end of pTAU-10 did not reach the NH_2-terminal end of the coding region. To obtain complete recombinants, we rescreened the same cDNA library by pTAU-10 and identified 66 positive phages. One of them, pTAU-16 (1,589 bp), reached the 5′ end and covered pTAU-10 completely. pTAU-16 contained a complete open reading frame of 1,296 residues (432 amino acids). Molecular mass calculated from the deduced amino acid sequence of pTAU-16 was 45,183 (Fig. 1 *A*). pTAU-10 corresponded with the nucleotide residues 83–1572 of pTAU-16. We also obtained another clone, pTAU-15 (1536 bp), which differed from pTAU-16 in the 5′ region, although it did not reach the 5′ end (Figs. 1 *A* and 2). Besides the deletion of 87 bp (29aa) upstream "A(92)EEA," the remaining sequence was completely the same with pTAU-16.

Northern blot of RNA obtained from an adult rat brain revealed that the cDNA hybridized to a single species of mRNA, which was surprisingly large in molecular mass (~6 kb) (Fig. 3). pTAU-16 had a complete open reading frame (1,296 bp) and the 3′-noncoding region (276 bp) that contained poly(A) tail with polyadenylation signal. Although we have not rigorously analyzed the structure of the 5′-noncoding region of the mRNA, this may indicate that its 5′-noncoding region, which is calculated as ~4.5 kb, occupies ~75% of the whole mRNA.

In Southern blot with the 3′ terminal half of pTAU-16 as a probe (the nucleotide residues 661–1210), the band on the lane appeared single when digested by PstI (Fig. 3). This is consistent with the idea that the tau mRNA is encoded by a single copy gene.

Tau Derived from Single cDNA Appears as Three Bands on SDS-PAGE but Becomes Two Bands after Phosphatase Treatment

Next, we transfected the tau gene to L-cells and studied the expressed tau proteins. To have the best chance of expressing tau in a variety of heterologous cells in tissue culture, we constructed a β-actin enhancer–driven plasmid, pβactTAU16, by exchanging the CAT gene of pβactCAT9 with pTAU-16 (Kost et al., 1983; Fregien and Davidson, 1986) and transfected pβactTAU16 to L-cells using the calcium phosphate method (Xie, 1984).

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Rat(16)  MAEPRQEFDT MEDQAG.... .......DYT MLQDQEGDMD HGLKESPPQP  39
Rat(15)            ( not reached to the 5' end)            PQP
Mouse    MADPRQEFDT MEDHAG.... .......DYT LLQDQEGDMD HGLK......
Human    MAEPRQEFEV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLK......

Rat(16)  PADDGSEEPG SETSDAKSTP TAEDVTAPLV EERAPDKQAT AQSHTEIPEG  89
Rat(15)  PADDGSEEPG SETSDAKSTP TAE.......  ..........  ..........
Mouse    ..........  ..........  ..........  ..........  ..........
Human    ..........  ..........  ..........  ..........  ..........

Rat(16)  TTAEEAGIGD TPNMEDQAAG HVTQARVAGV SKDRTGNDEK KAKGADGKTG 139
Rat(15)  ..AEEAGIGD TPNMEDQAAG HVTQARVAGV SKDRTGNDEK KAKGADGKTG
Mouse    ..AEEAGIGD TPNQEDQAAG HVTQARVA.. SKDRTGNDEK KAKGADGKTG
Human    ..AEEAGIGD TPSLEDEAAG HVTQARMVSK SKDGTGSDDK KAKGADGKT.
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Figure 2. NH_2-terminal variation in adult rat tau cDNAs and among species. Comparison of NH_2-terminal domain (1–139) of deduced amino acid sequence of rat tau cDNAs (pTAU-16 and pTAU-15) and fetal mouse and both fetal and adult human cDNAs (Lee et al., 1988; Goedert et al., 1988, 1989). "RAT(16)" and "RAT(15)" show the deduced sequences from pTAU-16 and pTAU-15 respectively. Both fetal and adult human tau cDNAs are completely identical except the insertion in the repeat sequence (Goedert et al., 1988, 1989). Asterisks show the positions of varied amino acid and ellipses show the deletions of amino acids. Besides the insertion in the repeat region, the COOH-terminal domain downstream the "A(92)EEA" was highly conserved (data not shown). In the NH_2-terminal domain, both mouse and human tau lacked 58 amino acids (34–91), and rat(15) also lacked the second half of this deletion (29aa, 63–91). It suggests that tau proteins have varied NH_2-terminal domain.

It is well known that tau makes multiple bands on SDS-PAGE, but why the "multiple bands" appear is unsettled (Cleveland et al., 1977*a*,*b*; Lindwall and Cole, 1984; Binder et al., 1985). As we used tau cDNA from rat brain, we checked the splitting of bands on SDS-PAGE of rat brain tau for the control, and found six bands of 63,000, 61,000, 58,000, 54,000, 52,000, and 49,000, respectively (Fig. 4 *A*). To correlate the tau derived from pTAU-16 with these six bands, we examined tau proteins extracted from the stably transfected L-cells with pβactTAU16 by immunoblot.

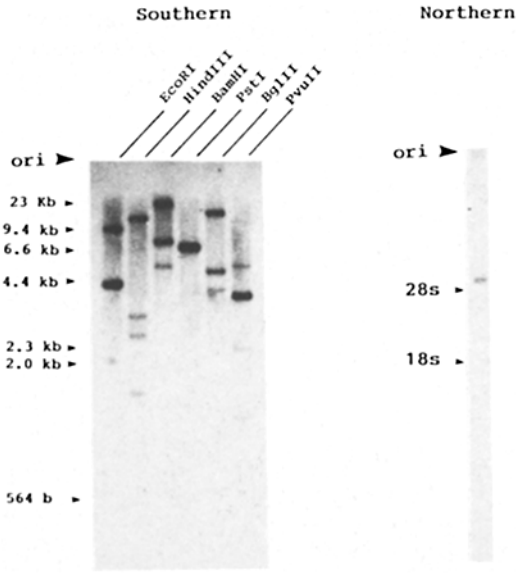


Figure 3. Southern and Northern blots. (*A*) Genomic Southern blots. Rat genomic DNA was digested by Eco RI, Hind III, Bam HI, Pst I, Bgl II, and Pvu II. The probe used was COOH-terminal half of pTAU-16 (the nucleotide fragment numbered 661–1,210). The band on the lane appeared single when digested by Pst I. (*B*) Northern blot. Total RNA was extracted from adult rat brain, and poly(A) RNA was purified by oligo(dT)-cellulose and fractionated in agarose gels (Maniatis et al., 1982). The same fragment used in Southern blot was chosen for probe. It shows that the cDNA hybridized to a single band (~6 kb). We used Gene Screen Plus filter (Du Pont Co., Wilmington, DE) for Genomic Southern blot and filter for Northern blot (Biodyne transfer membranes; Pall Corp., Glen Cove, NY). Hybridizations were performed following their standard protocols.

Microtubule preparations from the same amount of both stably transfected and native L-cells (~10^7) were obtained by the taxol method (Vallee, 1982). Both samples were subjected to SDS-PAGE (Fig. 4 *A*) and immunoblot (Fig. 4 *B*). In the immunoblot using anti-tau antibody, tau from a single cDNA origin appeared as three bands (Fig. 4 *B*, *b*). These three bands corresponded to the first, second, and fifth of the six bands of rat brain tau. The separation of bands in SDS-PAGE of tau derived from a single cDNA showed that nascent tau can be modified by posttranslational modification(s) into at least three molecular forms. It is well known that phosphorylation affects the mobility of tau (Lindwall and Cole, 1984). Therefore, we compared the SDS-PAGE mobility before and after treatment with E. coli alkaline phosphatase (Fig. 4 *C*). When tau from transfected L-cells was dephosphorylated, the first band disappeared, the second band became dense, and the fifth band did not change. The corresponding band in the rat brain tau showed the same changes through phosphatase treatment. As for the remaining bands, the third band became faint, the fourth band became dense, and the sixth band did not change. These changes of the first, second, and fifth bands were similar to those of third, fourth, and sixth bands, respectively. This suggests that one of the reasons of the diversity of tau bands on SDS-PAGE was the phosphorylation process.

Tubulin Increases in the Stably Transfected L-cells

It is well known that tau interacts with tubulin and enhances its polymerization into microtubules. Therefore, we also examined tubulin in the stably transfected L-cells. Interestingly, in the transfected L-cells, the bands of both α- and β-tubulin in the pellets of microtubule preparations appeared dense in SDS-PAGE (Fig. 4 *A*) and in immunoblot using anti-tubulin antibody (Fig. 4 *B*, *a*). To confirm the increase of tubulin, we quantitated a relative amount of tubulin in the crude extract. For this purpose we prepared microtubule proteins from the crude extract using taxol and then exam-

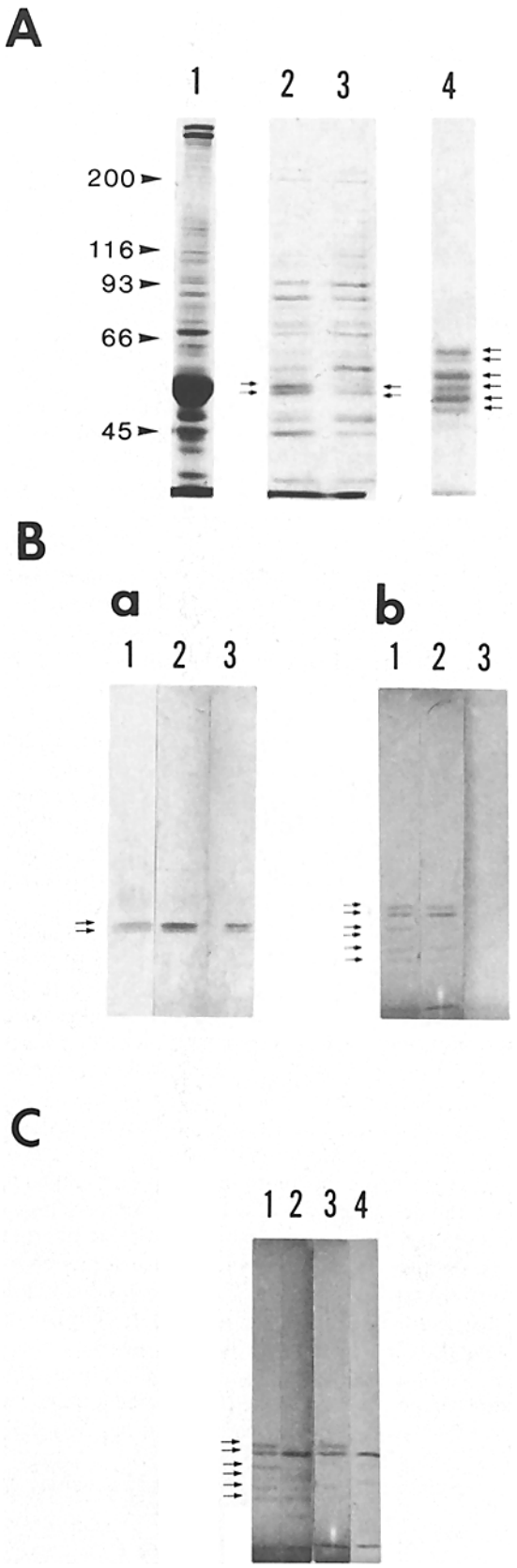


Figure 4. SDS-PAGE and immunoblot analysis. (*A*) Microtubule preparations from stably transfected and native L-cells obtained by the taxol method were examined by SDS-PAGE. Microtubule preparations from rat brain (lane *1*), from transfected L-cells (lane *2*), and from control native L-cells (lane *3*). Tau proteins from rat brain (lane *4*). Tau from rat brain revealed six bands (63,000, 61,000, 58,000, 54,000, 52,000, and 49,000, respectively, which were indicated by arrows in lane *4*). Tubulin increased in the transfected L-cells, which expressed tau (*arrows*, lanes *2* and *3*). (*B*) Immunoblot of transfected and native L-cells. Transfected and native L-cells were examined by immunoblot. In the lanes of transfected and native L-cells, half of the amount of microtubule preparations used in the SDS-PAGE were loaded. Antitubulin (*a*) and antitau (*b*). Microtubule preparations from rat brain as control (lane *1*), transfected L-cells (lane *2*), and native L-cells (lane *3*). Tau from transfected L-cells revealed three bands, although that from rat brain was six (*arrows*). Native L-cells did not express tau. The production of tubulin was enhanced in the transfected L-cells. Bands of α- and β-tubulin and tau are indicated by arrows beside the lanes. (*C*) Immunoblot of dephosphorylated tau. Immunoblot study of microtubule preparations from rat brain and from transfected L-cells before and after dephosphorylation. Untreated (lanes *1* and *3*) and *E. coli* alkaline phosphatase-treated (lanes *2* and *4*) microtubule preparations from rat brain (lanes *1* and *2*) and from transfected L-cells (lanes *3* and *4*). Tau derived from single cDNA formed three bands on SDS-PAGE (first, second, and fifth bands). The first band disappeared by dephosphorylation, the second band became dense, and the fifth band did not change its density. The corresponding bands in microtubule preparations from rat brain showed the same changes after phosphatase treatment. As for the remaining bands, the third band became faint, the fourth band became dense, and the sixth band did not change. Six bands of tau were shown by arrows.

ined the amount of tubulin in the pellets and supernatants using densitometer. After incubation at 37°C for 30 min in the presence of 1 mM GTP with 20 μM taxol, the resulting pellets and supernatants were subjected to SDS-PAGE. Peak areas corresponding to tubulin in the pellets and supernatants were measured by densitometry, and were compared with the total peak areas of both pellets and supernatants. Fig. 5 shows the densitometric scans of SDS-PAGE of both the supernatant and the pellet of the stably transfected L-cells and the control L-cells. Arrowheads point to the peaks of tubulin. The ratio of tubulin in the crude extract was 13.8% in the transfected L-cells, while 6.9% in the control. It shows about a twofold increase in the amount of tubulin in the stably transfected L-cells.

The Transfected Gene Product, Tau, Forms Microtubules into Thick Bundles in L-cells

Tau, which is localized to neurons (Binder et al., 1985), promotes the polymerization of tubulin (Weingarten et al., 1975) and forms cross-bridges between microtubules in in vitro reconstruction study (Hirokawa et al., 1988*a*). On the other hand, microtubules are observed as fine filamentous networks spread in most of the cultured cells of nonneuronal origin and also in the case of native L-cells (Fig. 6 *e*). What will happen to microtubules and the cell structure when tau is induced to nonneuronal cells? This should be an interesting approach to analyze the function of tau in vivo.

To answer this question, we observed transiently transfected L-cells. 35 h after transfection, cells were fixed. To localize tau derived from pTAU-16, we used a monoclonal antibody, TAU1 (Binder et al., 1985). A rabbit polyclonal antiserum for tubulin was used in double immunofluorescence studies to identify microtubule networks.

Tau was transiently expressed in some of the transfected

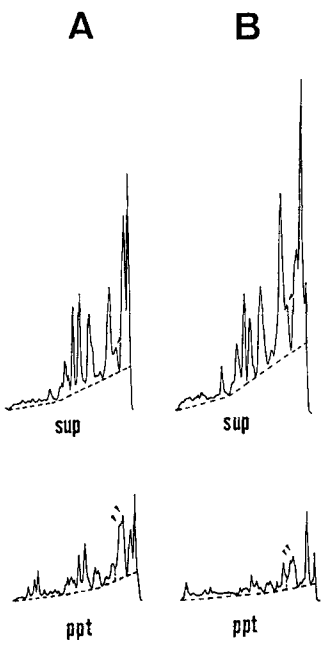


Figure 5. Densitometric scans of SDS gels of stably transfected and control L-cells. Same amounts of crude extracts of transfected and control L-cells were incubated at 37°C for 30 min with 1 mM GTP and 20 μM taxol, and were centrifuged at 10,000 *g* for 30 min. (*A*) Supernatant (*sup*) and pellet (*ppt*) of transfected L-cells and (*B*) supernatant and pellet of control L-cells. Arrows show the tubulin peaks both in pellets and supernatants, and the amounts of tubulin were measured in these areas. The ratio of tubulin to total proteins (supernatant and pellet) in the transfected L-cells was 13.8%, while that in the control was 6.9%. The amount of tubulin increased about twofold in the transfected L-cells.

L-cells, as assayed by staining with anti-tau antibody (Fig. 6, *a* and *b*). Transfection efficiency was ~5%. Next, to observe the localization of transfected tau proteins, cells were extracted by Triton X-100 and fixed. Interestingly, double-labeling experiments using anti-tau and anti-tubulin antibodies showed that microtubules tended to form thick bundles and anti-tau antibody mainly stained these bundles in the transfected cells (Figs. 6, *c–f*, and 7). The thickness of the bundles was 10–100 times that of normal microtubules and such bundles were never observed in the control cells. The number of thick bundles in a transfected cell was 20 at most, while that of normal microtubule filaments in a control cell was countless, no less than 1,000. Tau is localized in the axon, but not in the cell body nor in the dendrite when neuron was stained by anti-tau, TAU1 (Binder et al., 1985). On the other hand, the typical bundles in the transfected L-cells, which were also stained by TAU1, started from a cell projection, ran along the cell body and ended in another cell projection. Typical microtubule organization centers were not observed in these cells.

For further investigation of these bundles, we observed the transiently transfected L-cells in EM. Fig. 9 *a* shows a thick microtubule bundle in a process of a transfected cell. Fig. 9 *b* is the higher magnification. It is obvious that numerous microtubules form bundles in the process. The distance between microtubules varied, while most of them were <20 nm, which corresponded well with the distance observed between microtubules polymerized with tau in vitro (Hirokawa et al., 1988*a*). Sometimes the thickness of the bundle reached 2 μm. This data suggests that tau makes cross-bridges between microtubules in vivo, and corresponds to our previous in vitro reconstruction study (Hirokawa et al., 1988*a*). Other bundles were also shown in higher magnification (Fig. 9, *c* and *d*).

As for the stable cell lines, we could also observe bundles, although the number of bundles in a cell was mostly one, sometimes zero, while those in some transiently transfected cells were >10 (Figs. 6 *f*, 7, and 8).

Discussion

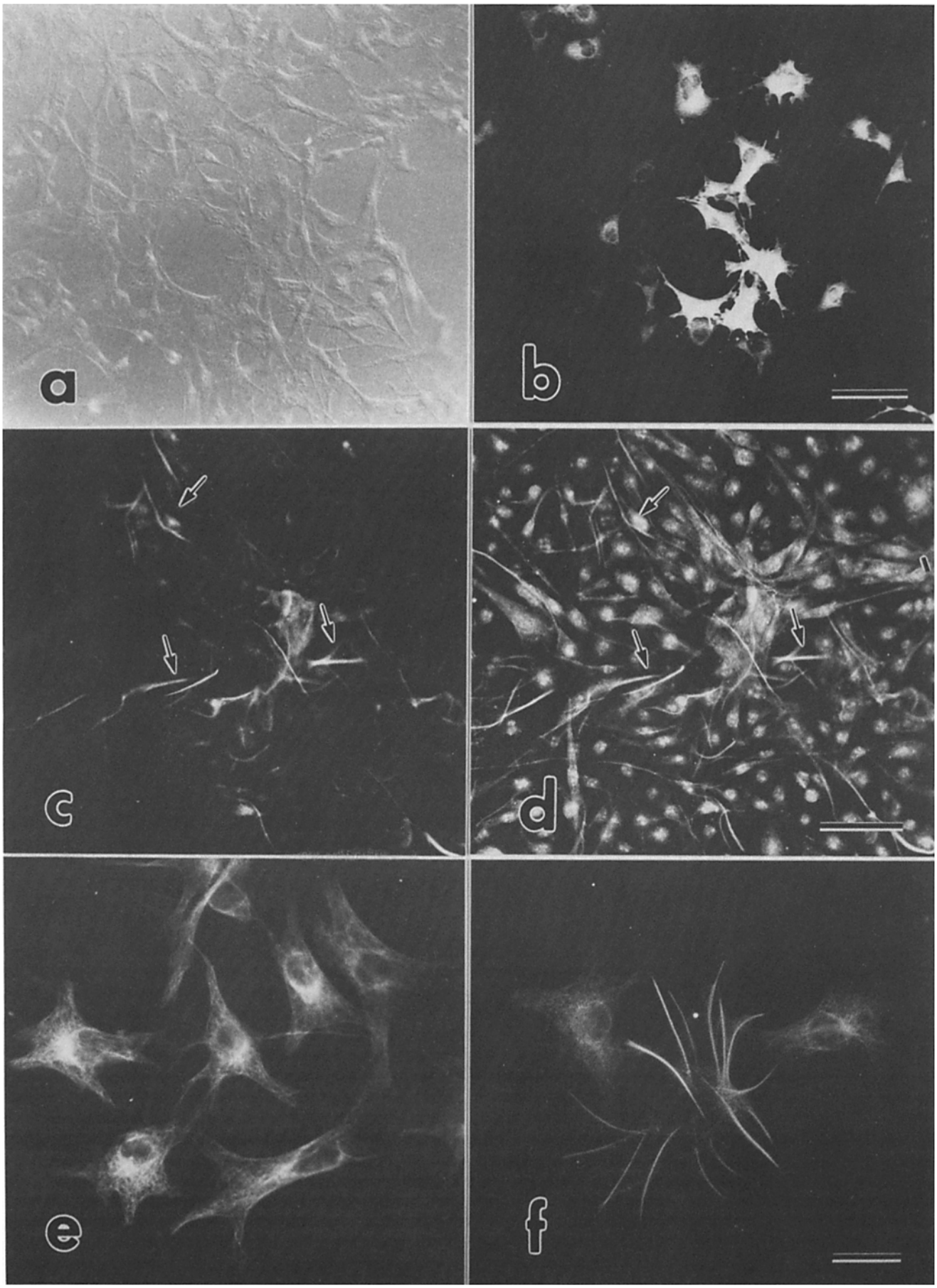
Primary Structure of Rat Tau cDNA

The cDNA encoded a polypeptide with 432 amino acid residues that contained four internal repeats of 18 amino acid residues within 112 amino acids located in the carboxyl half of the protein. Among the repeat units, the amino acids were highly conserved and 11 out of the 18 amino acids were completely identical (Fig. 1, *A* and *B*). We also isolated another cDNA (pTAU15) containing four repeat, which differed from pTAU16 at the 5′ region, although it lacked its 5′ end. Fig. 2 shows the NH_2-terminal differences of tau cDNAs of rat (pTAU16 and pTAU15) and other species tau already reported (Lee et al., 1988; Goedert et al., 1988, 1989). It is reported that there are three (juvenile) and four repeat (adult) tau isoforms and that the juvenile type lacks the second repeat unit of the adult type (Goedert et al., 1989) (Fig. 1 *A*). However, besides the insertion of the repeat unit, the COOH-terminal domain downstream the "A(92)EEA" sequence was highly conserved. As for the NH_2-terminal differences among species, it may be possible that the varied NH_2-terminal means the existence of subtypes in this region, although the case of deviation among species remains. pTAU15 lacked 29 amino acids (63–91), which was just the second half of the 58 amino acid sequence (34–91) not found in fetal mouse or either fetal or adult human tau sequences (Lee et al., 1988; Goedert et al., 1988, 1989) (Fig. 2). This reinforces the idea that varied NH_2-terminal isoforms may exist, perhaps by alternative splicing. Differences in both NH_2 and COOH-terminal suggest the possibility of functional changes of tau during development and/or differentiation.

There are reports that this repeating region is the binding site to microtubules (Aizawa et al., 1988; Lee et al., 1988). However, even if these repeats are binding sites to microtubules, to elucidate the phenomenon that tau forms cross-bridges between microtubules, our transfection study indeed showed that a single type of tau derived from pTAU-16 formed microtubule bundles, the distance between repeat units were too short to form the "bridge," and we must consider another hypothesis; e.g., tau has another binding site to tubulin in the NH_2-terminal end or two tau molecules form an antiparallel dimer.

In addition to the repeat sequence, tau had a unique se-

Figure 6. Immunofluorescence of transiently transfected L-cells. Low magnification observation of transiently transfected L-cells by Nomarski (*a*) and immunofluorescence using antitau antibody (*b*). Some of the L-cells expressed tau derived from pTAU-16. Double staining of Triton-extracted, transfected L-cells; antitau (*c*) and antitubulin (*d*). High magnification of antitubulin staining of native L-cells for the control (*e*) and transiently transfected L-cells (*f*). We found very thick bundles stained with both antitau antibody and antitubulin antibody in transiently transfected L-cells, although such bundles were not found in the control. Bars, (*a–d*) 100 μm; (*e* and *f*) 20 μm.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c
d
e
f

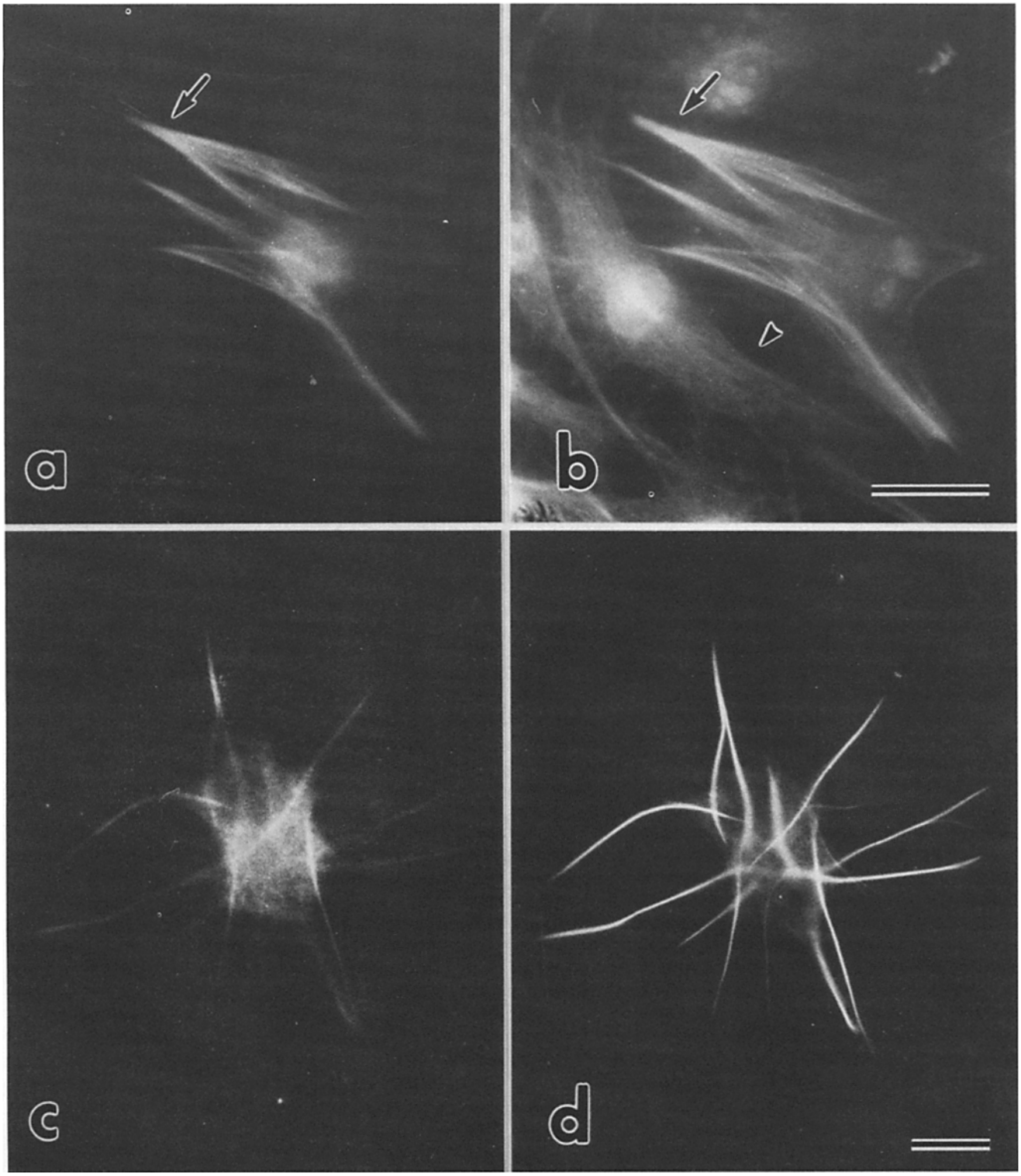

Figure 7. High magnification observation of transiently transfected L-cells. High magnification observation of transiently transfected L-cells with typical thick bundles using double staining with antitau (*a* and *c*) and antitubulin (*b* and *d*). In the transfected L-cells, microtubules formed thick bundles that were also stained with antitau antibody (*arrows*), while microtubules in the native L-cells remained as fine filaments (*arrowhead*). The normal microtubule organizing centers were not observed in the transfected cells. Bar, 20 μm.

quence of "Lys-Ser(387)-Pro-Val," which is also conserved in both mouse and human tau (Fig. 1 *A*). The "Lys-Ser-Pro-Val" sequence is also found 12 times in the repeating sequence of neurofilaments M and H (Napolitano et al., 1987; Geisler et al., 1987; Myers et al., 1987) (Fig. 1 *C*). The phosphorylation of the Ser residue within this repeating sequence in the neurofilament is thought to be profound, affecting both its physical properties and immunoreactivity (Myers et al.,

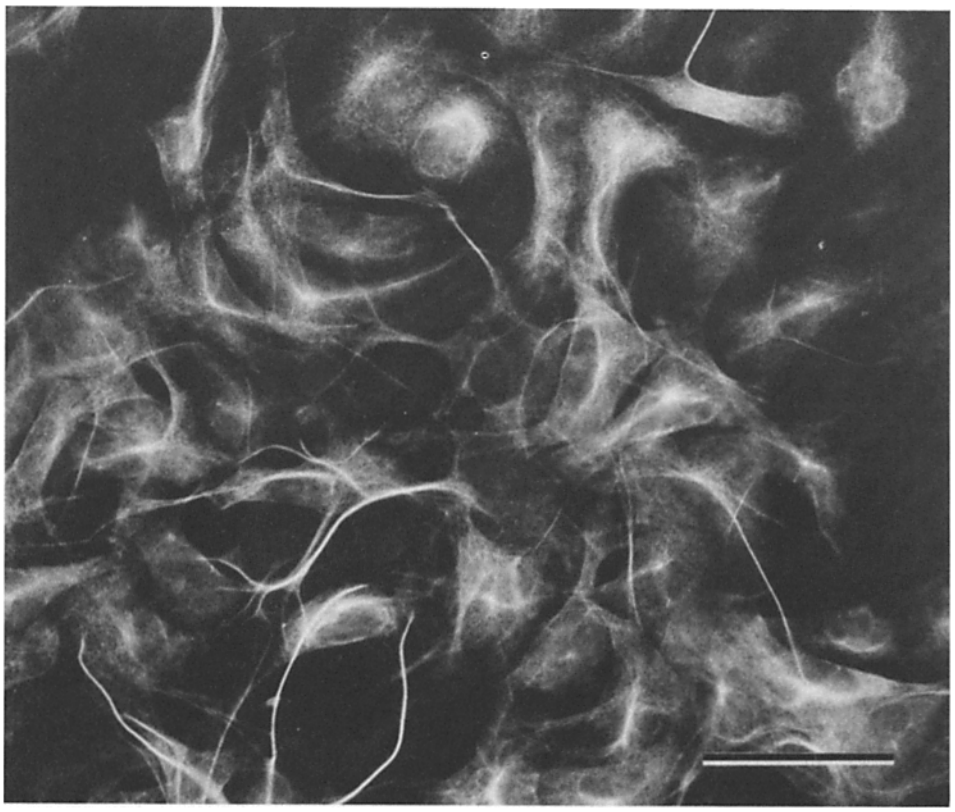

Figure 8. Antitau staining of stably transfected L-cells. Immunofluorescence of stably transfected L-cells with antitau antibody. This cell line was used for the SDS-PAGE and immunoblot in Figs. 4 and 5. Bundles of microtubules are observed, but they are not as many as that found in transiently transfected L-cells shown in Fig. 7. Bar, 100 μm.

1987). Tau also changes both its physical properties and immunoreactivity through phosphorylation. Phosphorylation affects the ability of tau to promote microtubule assembly and its mobility on SDS-PAGE, and the phosphorylated site that is involved in the regulation appears to be single (Lindwall and Cole, 1984). There was a report that some monoclonal antibodies to neurofilaments react with paired helical filaments in Alzheimer's disease (Nukina et al., 1987). These antibodies also recognize tau in the phosphorylated form, but do not react with that of the dephosphorylated form. In addition, this sequence is located near the repeating region, which is likely to be the binding site to tubulin. Therefore, it may be possible that the amino acid Ser-387 is the important phosphorylation site for the functioning of tau.

Why Does Tau Appear as Many Bands on SDS-PAGE?

Initially, we were surprised to discover that tau proteins derived from a single cDNA were separated into three bands on SDS-PAGE, and the indicated molecular masses were much higher than the predicted molecular mass from the primary structure. Tau from rat brain appeared as six bands, and the three bands derived from the cDNA corresponded to the first, second, and fifth of these six bands, respectively. In the dephosphorylation study, the first band disappeared and the second band became dense, but the fifth band did not change. The same changes occurred in the other three bands. Therefore, we think that the first band is the phosphorylated form of the second band, and, compared with these two bands, the fifth band is another modified form, including the possibility of a degradation product. As for the other three bands, like the bands derived from the cDNA, it may be possible that the third band is the phosphorylated form of the fourth band, and the sixth band is another modified form, although we have not determined their complete primary structure(s).

Our data shows that some kinds of posttranslational modifications, including phosphorylation, cause the splitting of tau on SDS-PAGE, but why the other three bands appeared in rat brain remains the next question, although even the three bands, to which pTAU-16 products corresponded, may contain other isoform types of tau. In mouse, there are at least two types of tau cDNA, one has a short and the other a long COOH-terminal domain (Lee et al., 1988). As for human tau, there is another type of classification; three repeat type (juvenile type, type I) and four repeat type (adult type, type II) (Goedert et al., 1989), and both of them are short type tau. Our rat tau cDNA was short and four repeat type. Besides the varied COOH-terminal domain, there are also the differences in the NH_2-terminal domain among species and within rat. These data suggest that another reason for the multiple band formation may be the difference of tau mRNA, perhaps long or short COOH-terminal type, juvenile or adult type tau mRNAs, and difference in the NH_2-terminal domain (Drubin et al., 1984; Lee et al., 1988; Goedert et al., 1989).

How Did Tau Influence the Microtubules Network?

Through the use of gene transfection, we observed a very interesting formational change of microtubules. It has been reported that tau enhances the polymerization of tubulin (Cleveland et al., 1977*a*,*b*) and forms cross-bridges between microtubules in vitro (Hirokawa et al., 1988*a*). Therefore,

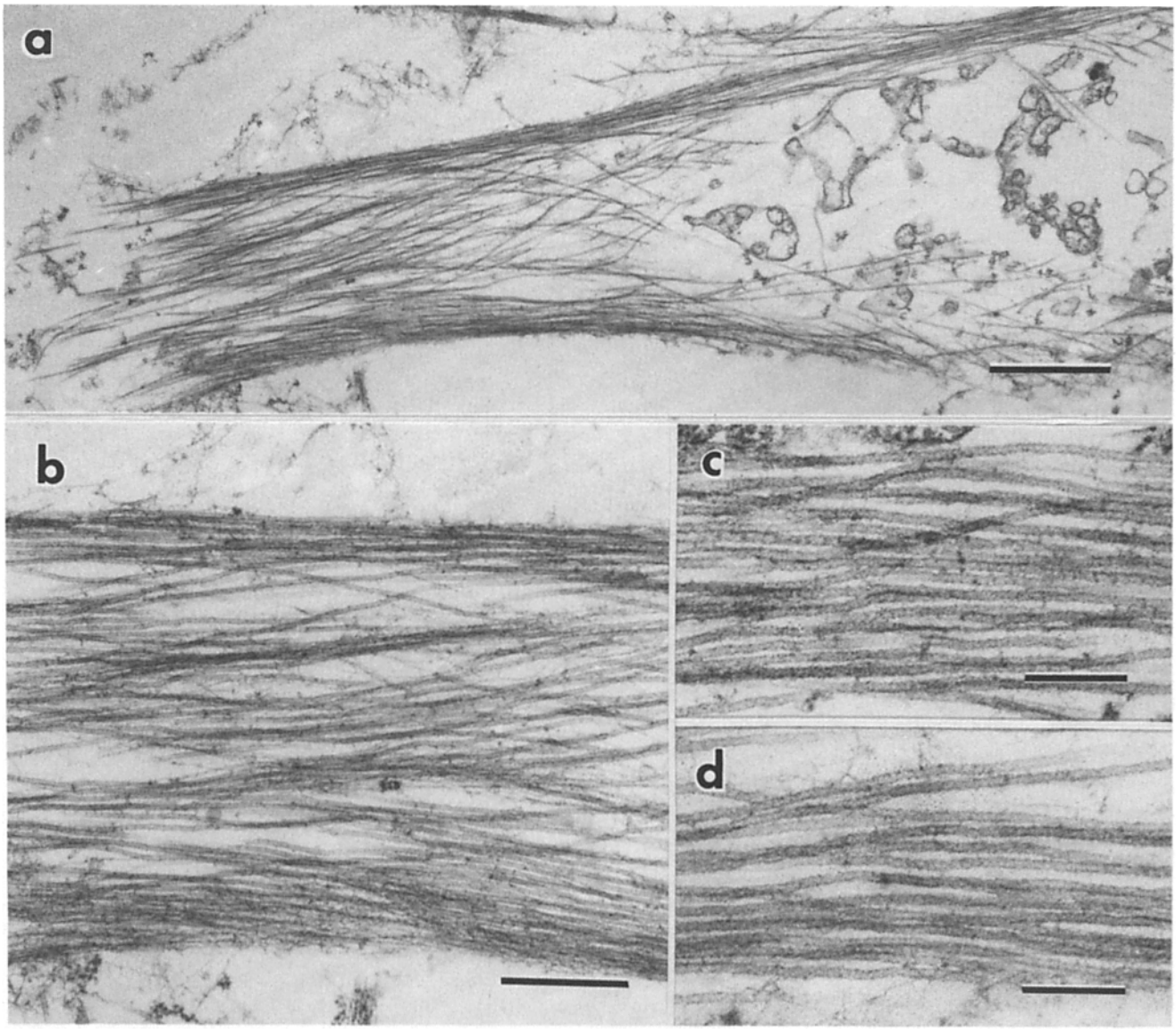

Figure 9. Electron micrograph of the L-cells transfected with pβactTAU16. Thick bundles of microtubules in a process of a fibroblast transfected with pβactTAU16 fixed after permeabilization in a microtubule stabilizing buffer (*a*). The thickness of this bundle was at least 2 μm. Bar, (*a*) 1 μm. Higher magnification: bar, (*b*) 0.5 μm. Two other bundles observed: bar, (*c* and *d*) 0.2 μm. Microtubules were bundled very tightly.

it is likely that, in the transfected cells, the expressed tau induced assembly of tubulin to microtubules and formed cross-bridges between microtubules, and consequently the thick bundles of microtubules were observed.

In our stable cell lines, we also found microtubule bundles, but the bundles were not as many as those observed in the cells with a typical bundle formation by transient transfection (Figs. 7 and 8). In the case of transiently transfected L-cells, it was not frequently found that cells with extremely thick bundles exist next to each other (Fig. 7). The transiently transfected L-cells were incubated for 35 h and totally increased about fourfold during this period. It is reported that the characteristic of transfected L-cells by the CaP technique is transmitted to 92% of the daughter cells (Xie, 1984). Therefore, it may be possible that most of the L-cells, which expressed a large amount of tau and formed many bundles, did not proliferate. It also suggests that division and proliferation were suppressed in the tau expressed L-cells, and only the cells that expressed a relatively low amount of tau could form stable cell lines.

There is a report that tau protein microinjected into fibroblast cells associates with microtubules, but does not cause major changes in the microtubule arrangement (Drubin and Kirschner, 1986). We think that the method of gene transfection induces a larger amount of tau into cells than the method of protein microinjection, and the high intracellular concentration of tau may be necessary for the bundle formation of microtubules. Therefore, our transient transfection of tau introduced enough of an amount of tau to L-cells to make microtubules into bundles.

The immunofluorescence and EM data strongly suggest that tau promotes tubulin polymerization and forms bundles of microtubules in vivo. Therefore, we think that tau plays an important role on the formation and maintenance of neu-

rites in nerve cells, which specifically express the protein. In fact, we observed by an in situ hybridization experiment that tau mRNA is highly expressed in the nerve cells of rat central nervous system at the stage when nerve cells elongate axons during development (Takemura et al., manuscript submitted for publication).

The Effect of Tau on Tubulin

In our immunofluorescence study of transiently and stably transfected L-cells, we observed very bright microtubule bundles when stained by antitubulin antibody. We compared the tubulin level in the stably transfected and native cells, and found about a twofold increase of both α- and β-tubulin in the transfected cells. Because a twofold increase of tubulin was observed in the stably transfected cells, it may be possible that much more of an increase of tubulin occurred in some transiently transfected cells such as cells in Fig. 7. There is a report that the free tubulin level controls the production of β-tubulin by translational regulation of mRNA stability (Yen et al., 1988). Unpolymerized tubulin subunits bind directly to the nascent aminoterminal tetrapeptide of β-tubulin. This binding is transduced through the adjacent ribosome to activate an RNase that degrades the ribosome-bound mRNA. Therefore, we think that the bundle formation of microtubules caused a decrease of free tubulin. This decrease of free tubulin inhibited the degradation of β-tubulin mRNA, and consequently, tubulin production increased.

We observed the increased density of tubulin bands but could not identify tau bands in the SDS-PAGE of the pellet fraction by taxol. Our previous study showed that the stoichiometry of tau versus tubulin in preparation of tau saturated microtubules from porcine brain was 1:~5 (molar ratio) (Hirokawa et al., 1988*a*). It means that the amount of tau was lower than one-fifth of the tubulin, and, therefore, we think the bands of tau were hidden behind other bands. As for the transient transfection, some L-cells had the opportunity to get many copies of tau cDNA, and expressed a large amount of tau. As a result, a lot of bundles of microtubules were formed (Fig. 7). In contrast, stably transfected L-cells did not produce enough of an amount of tau to make many bundles of microtubules that inhibited cell proliferation to kill themselves. We think that, for the stable cell lines transfected with tau gene, the production of exogenous gene must be relatively low compared with other kinds of stable cell lines whose productions are not lethal to themselves.

The tau gene-transfected fibroblasts could be a good model system to further characterize function of tau proteins for the morphogenesis of nerve cells.

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Note added in proof: Recently Kosik et al. also published adult rat tau sequence (1989. *Neuron.* 2:1389–1397).

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