Purification and Characterization of Assembly-Competent Tubulin from *Aspergillus nidulans*†

Yisang Yoon‡ and Berl R. Oakley*

Department of Molecular Genetics, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210

Received January 4, 1995; Revised Manuscript Received March 15, 1995®

ABSTRACT: We have developed a procedure for purifying assembly-competent tubulin from *Aspergillus nidulans*. To our knowledge, this is the first report of the purification of assembly-competent tubulin from a filamentous fungus, and the procedure should be of great value in analyzing the large number of α- and β-tubulin mutations that have been isolated and characterized in *A. nidulans*. Our procedure consists of overproduction of α- and β-tubulin, partial purification by ion-exchange chromatography, and final purification by rounds of assembly and disassembly. We have found that taxol promotes the assembly of *A. nidulans* tubulin into microtubules, but a higher concentration of taxol is required for maximal assembly of *A. nidulans* tubulin than is required for brain tubulin. The critical concentration for assembly in the presence of taxol is also significantly higher for *A. nidulans* tubulin than for brain tubulin. In addition, *A. nidulans* microtubules that were assembled and maintained in the presence of taxol depolymerized in conditions in which taxol-stabilized mammalian microtubules remain intact. These results suggest that *A. nidulans* tubulin has a lower affinity for taxol than mammalian tubulin.

Extensive genetic studies of microtubules and the genes that encode microtubule proteins have been carried out in the filamentous fungus *Aspergillus nidulans*. Genes for α- and β-tubulin were first identified in *A. nidulans* (Morris et al., 1979; Sheir-Neiss et al., 1978), and a large number of α- and β-tubulin mutations have been identified and partially characterized (Sheir-Neiss et al., 1978; Morris et al., 1979; Oakley & Morris, 1980, 1981; Gambino et al., 1984; Weatherbee & Morris, 1984; Oakley et al., 1985, 1987a). Microtubule biochemistry in *A. nidulans* has been limited, however, by the lack of a system for purifying functional microtubule proteins.

Functional microtubule proteins are usually purified by repeated cycles of assembly and disassembly of microtubules *in vitro*. A major problem, however, in assembling microtubule proteins from lower eukaryotes *in vitro* is the low tubulin concentration in the cytoplasm. Previous attempts at *in vitro* microtubule assembly with partially purified *A. nidulans* mycelial extracts were unsuccessful. Davidse and Flach (1977) found that a tubulin-like protein was sedimentable after incubating a diethylaminoethyl (DEAE)[1] -purified extract at 37 °C. The temperature-dependent precipitation of tubulin, however, was not reversible by cold, Ca^{2+}, colchicine, or MBC, which suggested that microtubule assembly was not responsible for the precipitation of tubulin. Weatherbee and Morris (1984) partially purified *A. nidulans* tubulin using DEAE-cellulose chromatography but did not report attempting microtubule assembly.

We have attempted to overcome the problem of low tubulin concentration in fungal cytoplasm by overexpressing α- and β-tubulin in *A. nidulans*. We have succeeded in purifying assembly-competent material and have begun to analyze *A. nidulans* tubulin biochemically. We have found that although the assembly of *A. nidulans* tubulin into microtubules is dramatically enhanced by taxol, the critical concentration for assembly in the presence of taxol is significantly higher than that of brain tubulin. In addition, microtubules assembled from *A. nidulans* tubulin are cold-labile even in the presence of taxol. The research presented here is, to our knowledge, the first report of the purification of native, assembly-competent tubulin from a filamentous fungus. It presents the possibility of analyzing biochemically the wealth of tubulin mutations in *A. nidulans* and of defining the nature of taxol/tubulin interactions with great precision.

MATERIALS AND METHODS

Strains. FGSC4 (Glasgow wild type) was used as a wild-type *Aspergillus nidulans* strain. *A. nidulans* strains Y12 (*pyr*G89, *pyro*A4, *y*A2) and G191 (*paba*A1, *pyr*G89, *fw*A1, *ua*Y9) were used as hosts for *Aspergillus* transformations. Y12 was constructed in our lab by crossing G191 and LO235 (*y*A2, *pyro*A4, *mip*B1). *Escherichia coli* strain JM109 was used for bacterial transformations.

Plasmids and Construction of a Tubulin Overexpression System. Plasmid pAL3 was previously constructed by Waring et al. (1989). It contains 380 bp of the *A. nidulans alc*A (alcohol dehydrogenase) promoter sequence in front of a polylinker. pAL3 also contains the *Neurospora crassa* gene *pyr*4 as a selectable marker. The *pyr*4 gene encodes orotidine 5′-phosphate decarboxylase and can complement the *pyr*G89 mutation of *A. nidulans* (Ballance et al., 1983), but because of its low sequence identity with the *pyr*G gene,

† Supported by Grant GM31837 from the NIH and Grant MCB-9222988 from the NSF.

* Author to whom correspondence should be addressed.

‡ Present address: Center for Basic Research in Digestive Diseases, Mayo Clinic, Rochester, MN 55905.

® Abstract published in *Advance ACS Abstracts,* May 1, 1995.

[1] Abbreviations: DEAE, diethylaminoethyl; MBC, methylbenzimidazol-2-yl carbamate; FGSC, Fungal Genetics Stock Center; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTP, guanosine 5′-triphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; DMSO, dimethyl sulfoxide; MAP, microtubule-associated protein.

0006-2960/95/0434-6373$09.00/0

it does not cause integration at the *pyr*G locus during transformation. Plasmid pALT6, which contains the *ben*A, β-tubulin, gene under the control of the *alc*A promoter (Waring et al., 1989), was provided by Dr. Gregory May (Baylor College of Medicine). Plasmid pDP485, which contains the *tub*A, α-tubulin, gene in pUC19 (Doshi et al., 1991), was obtained from Dr. Ronald Morris (Robert Wood Johnson Medical School).

To construct a plasmid that has the *tub*A, α-tubulin, gene under the control of the *alc*A promoter, we subcloned a *Bam*HI fragment from pDP485 into plasmid pAL3. The *Bam*HI fragment extends from 21 nucleotides upstream of the *tub*A start codon to well beyond the poly(A) addition site. We named the resulting plasmid pXTA2. We transformed *A. nidulans* strain Y12 with pXTA2 and, by Southern hybridization, identified a transformant carrying two copies of pXTA2 integrated at the *tub*A locus (Figure 1). We named this transformant Ypta2. We also transformed strain G191 with pALT6 and identified a transformant (Tpba2) containing two copies of pALT6 integrated at the *ben*A locus (Figure 1). We constructed a diploid strain, Ypta2/Tpba2, that carries α- and β-tubulin genes under the control of the *alc*A promoter in the same nucleus (Figure 1).

Media and Growth of Aspergillus nidulans. As a complete liquid medium, YG (5 g/L yeast extract, 20 g/L dextrose) was used. YAG and FYG were used as solid media. YAG is YG containing 15 g/L agar, and FYG is YG containing 25 g/L Pretested Burtonite 44c (TIC Gums, Inc., Belcamp, MD). Both YAG and FYG were supplemented with a trace element solution (Dr. C. F. Roberts, University of Leicester, personal communication). For regeneration of protoplasts after transformation, 1 M sucrose was added to YAG as an osmotic stabilizer. All cultures were grown at 37 °C. Liquid cultures were generally inoculated with conidia (asexual spores) at a density of $(1-2) \times 10^6$ conidia/mL of medium. Inducing medium for the *alc*A promoter consisted of 6 g/L $NaNO_3$, 0.52 g/L KCl, 1.52 g/L KH_2PO_4, 1 mL/L trace element solution, 0.052% (w/v) $MgSO_4 \cdot 7H_2O$, necessary nutritional supplements, and 50 mM methyl ethyl ketone (butan-2-one) as an inducer (Creaser et al., 1985). Since methyl ethyl ketone is not metabolized by *A. nidulans*, 4 mM fructose was added as a carbon source.

Transformation of A. nidulans and Southern Hybridization Analysis. A. nidulans strains Y12 and G191 were transformed with plasmid DNA using the procedure described by Oakley et al. (1987b). Minipreps of *A. nidulans* DNA and Southern hybridizations were carried out by the procedure of Oakley et al. (1987c).

SDS−PAGE and Western Blot Analysis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out using a buffer system based on the method of Laemmli (1970). For Western blotting, proteins were transferred without staining to a nitrocellulose filter using a PolyBlot apparatus (American Bionetics, Inc.) equipped with MilliBlot electrodes (Millipore). The nitrocellulose filter was blocked for 1 h with Tris-buffered saline with Tween (TBST: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) containing 5% nonfat dry milk. The blocked filter was incubated for 2 h with primary antibody diluted in TBST containing 1% nonfat dry milk. After the primary antibody reaction, the filter was washed in TBST for 15 min with three buffer changes and then incubated for 1 h with alkaline phosphatase-conjugated secondary antibody diluted in TBST. Before use, the secondary antibody was preadsorbed with an *A. nidulans* acetone powder for 2−4 h on ice to eliminate nonspecific reactions with *A. nidulans* proteins. The *A. nidulans* acetone powder was prepared according to the method described by Harlow and Lane (1988). After three 5 min washes with TBST, the filter was submerged in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 5 mM $MgCl_2$, and 100 mM NaCl) containing 3.3 mg/10 mL of NBT and 1.65 mg/10 mL of BCIP for color development. All incubations and washes were at room temperature.

DM1A (Sigma Chemical Co.), a monoclonal anti-α-tubulin antibody, and Tu27B, a monoclonal anti-β-tubulin antibody generously donated by Dr. Lester I. Binder (Molecular Geriatrics Corp.), were used as primary antibodies. Alkaline phosphatase-conjugated goat anti-mouse IgG (Hyclone) was used as the secondary antibody in all cases.

Purification of A. nidulans Tubulin. (1) Sample Preparation. The diploid strain Ypta2/Tpba2 was inoculated at 2×10^6 conidia/mL of medium and grown in 4 L of YG at 37 °C for 15 h. Mycelia were harvested and washed with sterile distilled water by filtration through Miracloth (Calbiochem). Washed mycelia were then transferred to 3.2 L of inducing medium containing 50 mM methyl ethyl ketone as an inducer. After induction for 9 h at 37 °C, mycelia were harvested by filtration through Miracloth and washed with cold distilled water. Mycelial yield was about 40−50 g wet weight. Mycelia were quickly frozen in liquid nitrogen and pulverized by cryo-impaction (Smucker & Pfister, 1975) for 2.5 min. The finely ground, frozen mycelial powder was resuspended in 50−60 mL of extraction buffer (100 mM MES, pH 6.6, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.2% Triton X-100) plus 0.1 mM GTP, protease inhibitors (1 mM PMSF and 5 μg/mL leupeptin), and nuclease solution (40 μg/mL RNase A and 20 μg/mL DNase I). After incubation on ice for 20 min, cell debris was spun down at 120000*g* for 30 min in a Beckman 60Ti rotor, and the clear supernatant was used as a sample for DEAE-cellulose chromatography.

(2) DEAE-Cellulose Column Chromatography. A DEAE-cellulose (Whatman DE52) column was equilibrated with MEM buffer (100 mM MES, pH 6.6, 1 mM EGTA, and 0.5 mM $MgCl_2$) containing 0.2 M NaCl, 0.1 mM GTP, and 0.1 mM PMSF. The sample was applied to a DEAE-cellulose column (2.5 cm × 8 cm) using a flow adaptor (Bio-Rad) and washed with MEM plus 0.2 M NaCl, 0.1 mM GTP, and 0.1 mM PMSF until no proteins came out of the column. After the column was washed, proteins bound to the column were eluted with MEM buffer containing 0.4 M NaCl, 0.1 mM GTP, and 0.1 mM PMSF. The direction of flow during elution was opposite to the direction of flow during application.

(3) Sephadex G-25 Column Chromatography. Gel filtration chromatography was used for desalting and buffer exchange. To precipitate proteins, 2 volumes of PEM (100 mM PIPES, pH 6.9, 1 mM EGTA, and 1 mM $MgSO_4$) saturated with ammonium sulfate were added to the protein eluted from the DEAE-cellulose column, and the mixture was incubated on ice for 20 min. After centrifugation at 15 000 rpm in a Beckman JA-20 rotor (18000*g*) for 15 min, the protein pellet was resuspended in PEM to a final volume of 1−2 mL. This sample was loaded onto a Sephadex G-25 column (1.0 cm × 18 cm) equilibrated with PEM plus 0.1

mM GTP and 0.1 mM PMSF, and proteins were eluted with the same buffer.

The desalted protein solution was cleared by ultracentrifugation for 30 min at 4 °C in a Beckman 50Ti rotor at 110000*g*, and the clear supernatant was drop-frozen in liquid nitrogen and then stored at −70 °C.

(4) Temperature-Dependent in Vitro Assembly and Disassembly of Tubulin. The frozen desalted protein was thawed and spun for 30 min at 4 °C in a Beckman 50Ti rotor at 110000*g* to clear the protein solution. The clear supernatant was made to 1 mM GTP, 50 μg/mL leupeptin and then incubated at 32 °C for 20 min. The sample was then centrifuged in a Beckman 70.1Ti rotor at 110000*g* for 20 min at 32 °C. The pellet was resuspended in PEM (a half volume of the initial solution) and incubated on ice for 40 min to allow depolymerization. The sample was then centrifuged in a Beckman 50Ti rotor at 56000*g* for 20 min at 4 °C. The second round of assembly and disassembly was a repeat of the procedure used for the first round except that leupeptin was omitted.

Purification of Brain Tubulin. Brain microtubule protein was prepared by a slight modification of the method of Shelanski et al. (1973). Bovine brain tissue, 100 g at a time, was homogenized at 4 °C in 75 mL of PEM containing 4 M glycerol. The homogenate was centrifuged at 14 000 rpm (30000*g*) in a Beckman JA-14 rotor for 20 min at 4 °C. The supernatant was centrifuged again at 120000*g* in a Beckman Ti60 rotor for 60 min at 4 °C. The supernatant from the ultracentrifugation was made to 0.5 mM GTP and incubated at 37 °C for 30 min to assemble microtubules. Polymerized microtubules were spun down at 120000*g* in a Beckman Ti60 rotor for 45 min at 37 °C. The microtubule pellet was resuspended in 4−5 pellet volumes of cold PEM buffer containing 0.5 mM GTP and chilled on ice for 30 min to disassemble microtubules. The disassembled protein solution was centrifuged at 90000*g* in a Beckman Ti60 rotor for 30 min at 4 °C. The second cycle of assembly and disassembly was the same as the first cycle except that glycerol was omitted in the assembly step.

A phosphocellulose (PC) column was used for removing microtubule-associated proteins from brain microtubule proteins which had been prepared by two cycles of assembly and disassembly of microtubules. The PC (Whatman P11) column was equilibrated with PEM buffer containing 0.1 mM GTP. Twenty to thirty milligrams of microtubule protein from bovine brain was loaded onto a PC column (1.0 cm × 20 cm), and PC-tubulin was eluted with PEM buffer plus 0.1 mM GTP.

Protein Determination. Protein quantitation was performed by the BCA method (Pierce) using bovine serum albumin as a standard.

Electron Microscopy. Samples were fixed with 0.2% glutaraldehyde, and 5 μL of fixed samples was placed on Formvar-coated copper grids (200 or 400 mesh, Electron Microscopy Sciences) for 10−20 s. After the grids were washed with water, three drops of 2% uranyl acetate were added for negative staining. Grids were examined in a Zeiss 10 electron microscope at 80 kV.

RESULTS

Overproduction and Partial Purification of Tubulin. Repeated attempts to obtain assembly of microtubule proteins from *A. nidulans* were unsuccessful, perhaps due to the low concentration of microtubule proteins in *A. nidulans* hyphae. We consequently attempted to overproduce α- and β-tubulin by creating strains in which α- and β-tubulin genes are under the control of the highly inducible *alc*A promoter (Creaser et al., 1985; Doy et al., 1985; Gwynne et al., 1987).

Since tubulin exists as a heterodimer in cells, unpaired monomeric α- and β-tubulin molecules resulting from the separate overexpression of α- and β-tubulin might be susceptible to proteolytic degradation or subject to some other regulatory mechanism to eliminate any excess of unpaired monomers. In addition, overexpression of β-tubulin alone in *Saccharomyces cerevisiae* is highly toxic to the cell (Burke et al., 1989; Weinstein & Solomon, 1990), and induction of two copies of the *A. nidulans ben*A, β-tubulin, gene under the control of *alc*A promoter has been reported to inhibit growth (Waring et al., 1989). We reasoned that overexpression of both α- and β-tubulin in the same cytoplasm might overcome these potential problems and produce more functional heterodimers. To create a strain capable, in principle, of simultaneously overexpressing α- and β-tubulin, we constructed a diploid from Ypta2 and Tpba2. This diploid carries two inducible *tub*A and *ben*A genes as well as two copies of *tub*A and *ben*A under the control of their normal promoters (Figure 1).

We tested overproduction of tubulin protein by Western blotting. As expected, in two control strains (the wild-type haploid FGSC4 and a diploid made from strains R153 and R21) in which the *tub*A and *ben*A genes are under control of their normal promoters, we found no increase in tubulin levels under inducing conditions (results not shown). In repeated experiments, we also failed to detect any increase in the level of α-tubulin in Ypta2 or the β-tubulin level in Tpba2 after 8 or 9 h of induction (results not shown). In the Ypta2/Tpba2 diploid, however, we consistently found a 2−4-fold increase in α- and β-tubulin levels after the same period of induction (Figure 2A). Note: the same amount of total protein (20 μg) was loaded in each lane in Figure 2A. Although the increase in the tubulin concentration after induction was small, we have consistently had much better results in purifying assembly-competent tubulin from the Ypta2/Tpba2 diploid, induced to overexpress tubulin, than from wild-type strains or the same diploid under noninducing conditions. We have consequently used induced Ypta2/Tpba2 as the source for tubulin for the experiments reported here.

Surprisingly, Northern blot analyses indicated that levels of α-tubulin mRNA in Ypta2 and the Ypta2/Tpba2 diploid dramatically increased upon induction, reaching, after 8 h of induction, levels 50−100-fold greater than the uninduced level (results not shown). β-Tubulin mRNA levels were similarly elevated in Tpba2 and the Ypta2/Tpba2 diploid. These results indicate a strong posttranscriptional regulation of tubulin levels in *A. nidulans*, a phenomenon that is currently under investigation.

The fact that tubulin is acidic allows it to be partially purified, rapidly and easily, using anion-exchange columns (Murphy & Borisy, 1975; Jacobs & Huitorel, 1979; Kilmartin, 1981; Weatherbee & Morris, 1984). We applied extracts from the induced Ypta2/Tpba2 diploid to a DEAE-cellulose column, washed the column with 0.2 M NaCl, and eluted bound proteins with 0.4 M NaCl. As shown in Figure 2B,

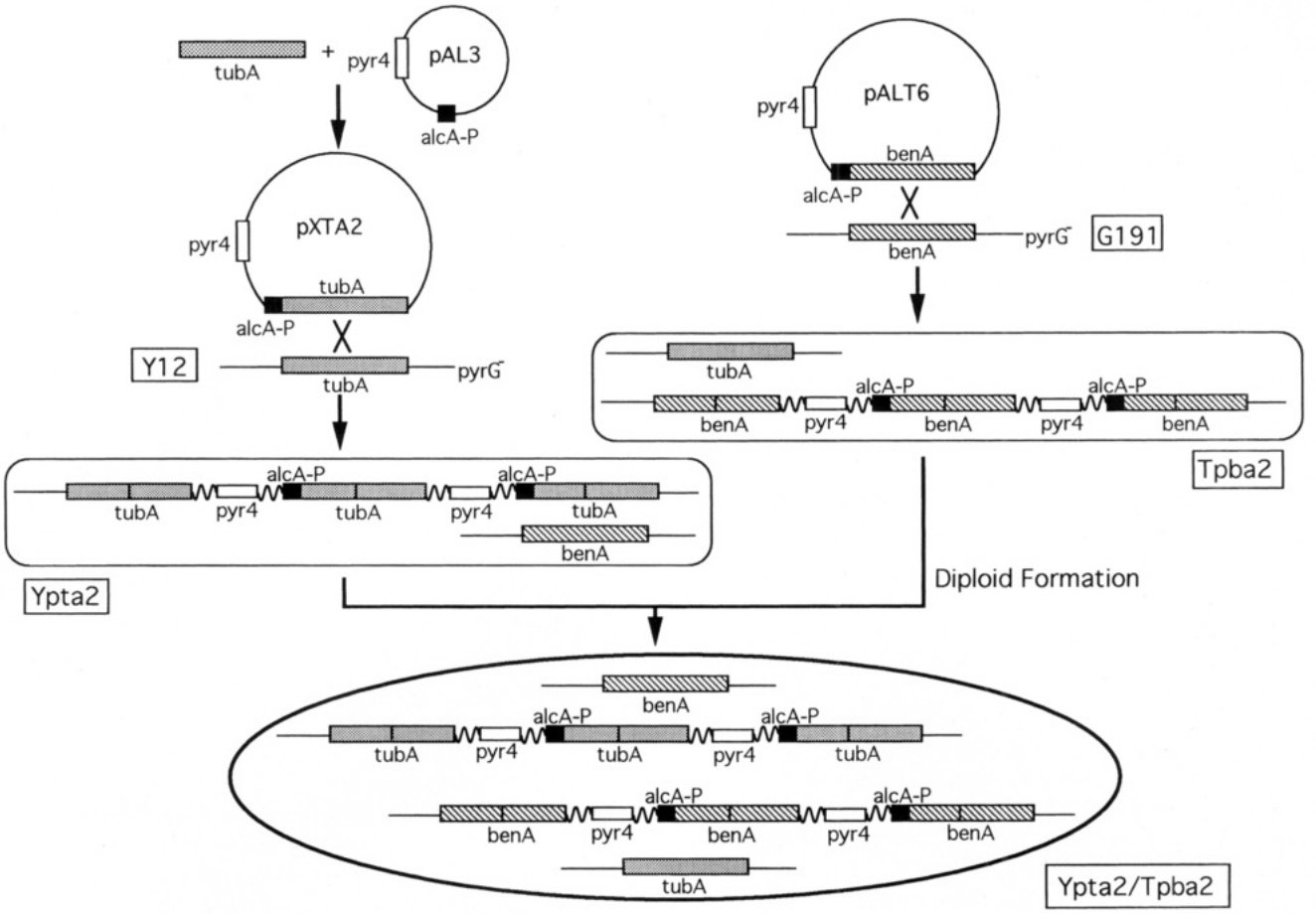

FIGURE 1: Schematic illustration of the construction of the *A. nidulans* tubulin overexpression system.

tubulin was enriched and appeared as two thick bands in the eluate. Western blotting confirmed that the two bands were α- and β-tubulin (results not shown).

Temperature-Dependent Assembly and Disassembly of Microtubules. We initially tried to assemble partially-purified *A. nidulans* tubulin in PEM plus 1 mM GTP. SDS−PAGE suggested that this procedure was successful. The great majority of the tubulin assembled into a sedimentable complex in the warm and disassembled in the cold. After two cycles of assembly and disassembly, we obtained relatively pure tubulin (lanes 9 and 10 in Figure 3A), along with a small amount of a 30 kDa polypeptide. In Western blots, the 30 kDa band did not react with polyclonal anti-tubulin antibodies, indicating that the 30 kDa polypeptide that copurified with tubulin was not a proteolytic product of tubulin (results not shown).

Electron microscopy showed, surprisingly, that the polymerized material was a mixture of some microtubules and many large aggregations of protofilaments (Figure 3B) that were mostly coiled (similar to the structures shown in Figure 6C). In many experiments, only protofilament aggregates were seen. The majority of the tubulin was thus apparently assembling into protofilaments rather than microtubules.

In an effort to find conditions that promoted the assembly of microtubules rather than protofilaments, we tested the effects of 2−10% DMSO (Himes et al., 1977), 4 M glycerol (Lee & Timasheff, 1975), 10 mM Mg^{2+} (Herzog & Weber, 1977), and brain MAPs. None effectively promoted microtubule assembly (results not shown). DMSO did increase the amount of sedimentable product after assembly reactions, but electron microscopy of material assembled with 2−10% DMSO showed an increased formation of protofilament aggregates and almost no microtubules (results not shown). These results suggest that DMSO promotes assembly of purified *A. nidulans* tubulin into protofilament aggregates rather than microtubules under the conditions employed.

Taxol Promotes Assembly of Aspergillus nidulans Tubulin into Microtubules. Taxol has been used to promote assembly of tubulin into microtubules from a wide variety of organisms (Vallee, 1982; Morejohn & Fosket, 1982; Vallee & Bloom, 1983; Collins & Vallee, 1987; Bokros et al., 1993). Even a low concentration of taxol (5 μM) not only promotes the assembly of mammalian tubulin at very low concentrations but also stabilizes assembled microtubules (Schiff et al., 1979; Kumar, 1981).

To determine if taxol affected the forms of polymer into which *A. nidulans* tubulin assembled, we added various concentrations of taxol to a 1.1 mg/mL tubulin solution. Surprisingly, 2−20 μM final concentrations of taxol were not effective in promoting assembly of *A. nidulans* tubulin into microtubules. At these concentrations, electron microscopy showed that polymerized products were mainly large protofilament aggregates (results not shown). At 40 μM taxol, however, microtubules were the main product although some aggregates of protofilaments remained. Above 60 μM taxol, only microtubules were found (Figure 3C).

To determine if taxol promotes *A. nidulans* tubulin polymerization, we assembled four different concentrations of *A. nidulans* tubulin and two concentrations of brain PC-tubulin in the presence of various concentrations of taxol

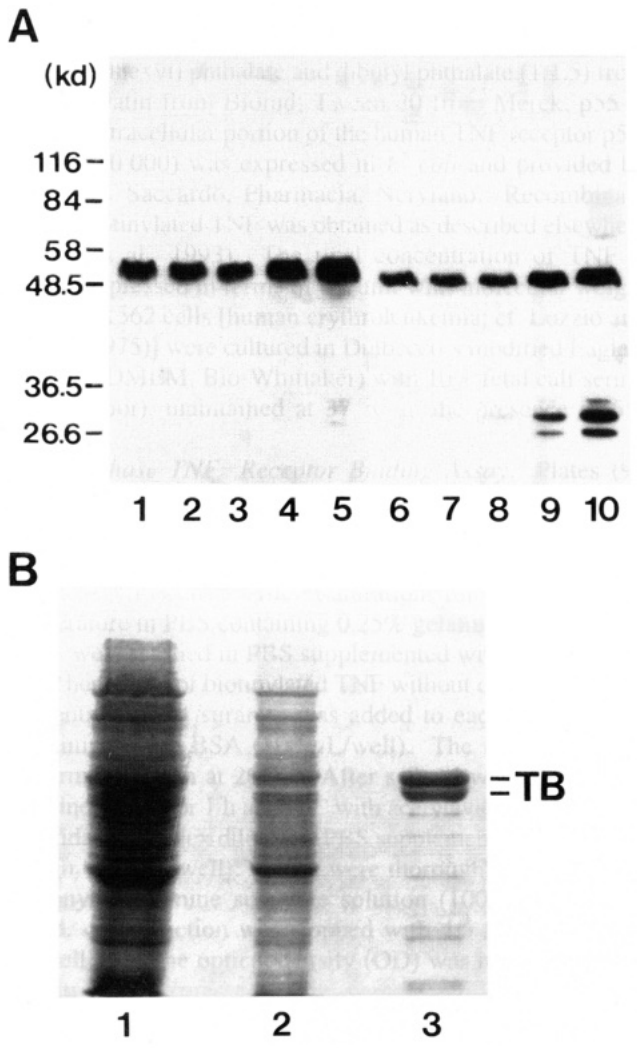

FIGURE 2: (A) Western blot analyses of total proteins from the Ypta2/Tpba2 diploid. The diploid cells were first grown in YG for 15 h and then transferred to inducing medium. Total protein (20 μg/lane) was extracted from uninduced cells (grown in YG; lanes 1 and 6) and cells induced for 1 h (lanes 2 and 7), 2 h (lanes 3 and 8), 4 h (lanes 4 and 9), and 8 h (lanes 5 and 10). α-tubulin was detected with antibody DM1A (lanes 1−5), and β-tubulin was detected with antibody Tu27B (lanes 6−10). Both α-tubulin and β-tubulin are overexpressed upon prolonged induction. Low molecular weight bands which we presume to be proteolytic cleavage products of α- and β-tubulin are found upon prolonged induction, and a faint unexplained higher molecular weight band was consistently recognized by the β-tubulin antibody after long inductions. (B) Coomassie blue-stained gel of samples from the partial purification of *A. nidulans* tubulin. Total extract (lane 1) of the Ypta2/Tpba2 diploid induced for 9 h was applied to a DEAE-cellulose column. The column was first eluted with 0.2 M NaCl (lane 2) and then eluted with 0.4 M NaCl (lane 3). The two prominent bands in lane 3 are α- and β-tubulin.

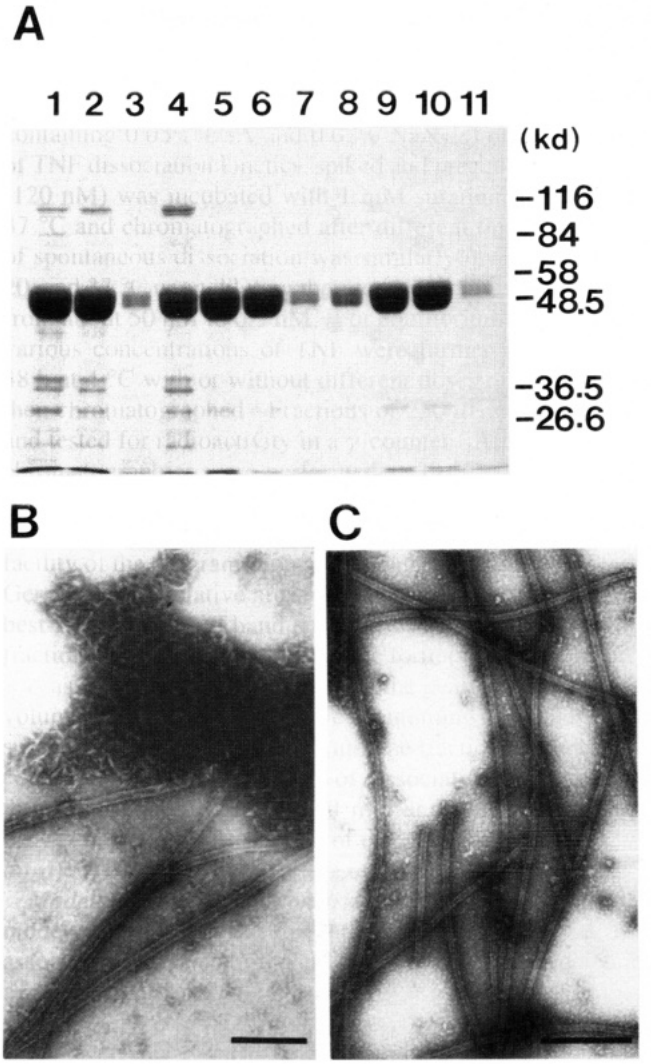

FIGURE 3: (A) Coomassie blue-stained gel of samples from the purification of tubulin by two rounds of assembly and disassembly. Lane 1, partially purified and desalted protein; lanes 2 and 3, supernatant and pellet after a cold spin to clarify the solution after thawing; lanes 4 and 5, supernatant and pellet after the first assembly; lanes 6 and 7, supernatant and pellet after the first disassembly; lanes 8 and 9, supernatant and pellet after the second assembly; lanes 10 and 11, supernatant and pellet after the second disassembly. (B) Electron micrograph of the second-round assembly product equivalent to lane 9 of (A) (scale bar: 0.2 μm). Although this field shows several microtubules, protofilament aggregates were much more numerous than microtubules in the original specimen. (C) Electron micrograph of *A. nidulans* microtubules assembled in the presence of 60 μM taxol (scale bar: 0.4 μm).

(Figure 4). In the brain PC-tubulin control, a low taxol concentration was sufficient to obtain maximal microtubule assembly (Figure 4B). The effect of taxol on *A. nidulans* tubulin was less dramatic (Figure 4A). Taxol increased the fraction of *A. nidulans* tubulin that assembled into sedimentable polymer, but the effect was less pronounced than with brain tubulin and maximum enhancement of polymerization occurred at 80 μM taxol or higher, much higher than the concentration needed for maximal enhancement of brain tubulin polymerization. It thus appears that, at high concentrations, taxol has a relatively small effect on the assembly of *A. nidulans* tubulin into polymer but causes a dramatic shift of the form of the polymer from protofilaments to microtubules.

We determined the critical concentration for the assembly of *A. nidulans* tubulin in the presence of 100 μM taxol (a concentration at which the assembly product is exclusively microtubules) in two ways. First, we measured the concentration of tubulin in the supernatant after microtubules were assembled and pelleted. The values, in taxol concentrations where maximal microtubule assembly occurred, were 0.16−0.20 mg/mL for *A. nidulans* tubulin (Figure 5) and approximately 0.05 mg/mL for brain PC-tubulin (results not shown). Second, we assembled *A. nidulans* tubulin and brain PC-tubulin at different concentrations in the presence of 100 μM and 20 μM taxol, respectively, and measured the fractions that assembled into microtubules. When the tubulin

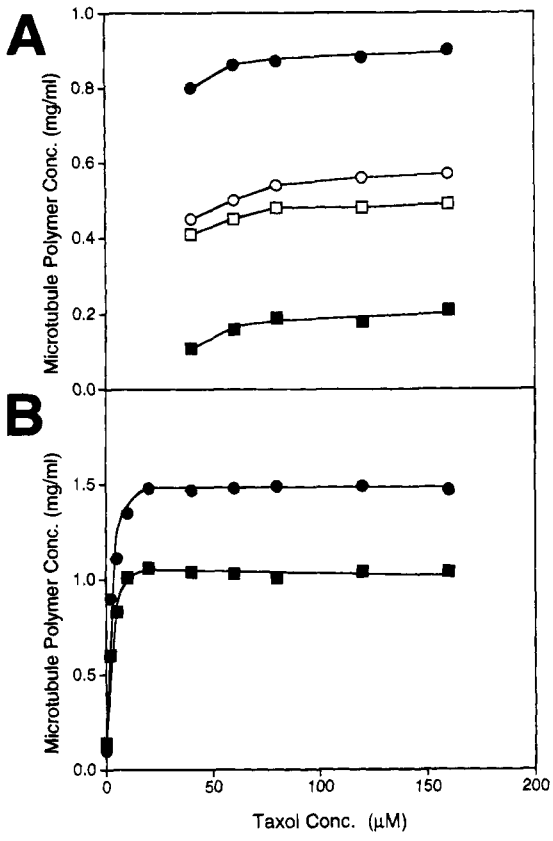

FIGURE 4: Effects of taxol concentration on the assembly of *A. nidulans* tubulin and brain PC-tubulin. In each panel, the *y*-axis values are the amount of sedimentable tubulin polymer in each assembly mixture divided by the volume of the mixture. These values are plotted against the taxol concentrations in the assembly mixtures (*x*-axis). (A) The effect of taxol on the assembly of *A. nidulans* tubulin. 1.1 mg/mL (●), 0.75 mg/mL (○), 0.66 mg/mL (□), and 0.43 mg/mL (■) *A. nidulans* tubulin were incubated at 32 °C for 30 min in the presence of 40, 60, 80, 120, and 160 μM taxol and spun for 30 min at 110000*g*. Protein concentrations of each supernatant were measured and used to calculate the amount of tubulin in the pellet. This value was divided by the volume of the assembly mixture to obtain the values shown. Lower taxol concentrations were not used with the *A. nidulans* tubulin because the predominant form of polymer at low taxol concentrations is protofilaments rather than microtubules and even at the lowest taxol concentration in this experiments (40 μM) some protofilaments were present. (B) The effect of taxol on the assembly of brain PC-tubulin. 1.53 mg/mL (●) and 1.08 mg/mL (■) brain PC-tubulin were incubated at 37 °C for 30 min in the presence of 0, 2, 5, 10, 20, 40, 60, 80, 120, and 160 μM taxol and centrifuged at the incubation temperature for 30 min at 110000*g* and the microtubule polymer concentration was determined as for *A. nidulans* tubulin. Virtually all of the brain tubulin assembled into microtubules at concentrations of taxol of 20 μM or greater, but, as is shown in panel A, higher concentrations were required for maximal assembly of *A. nidulans* tubulin.

that assembled into microtubules (*y*-axis) is plotted with respect to the total tubulin in the assembly solution (*x*-axis), the critical concentration for assembly is the intercept on the *x*-axis. The critical concentration for *A. nidulans* tubulin was again approximately 0.16 mg/mL and that of brain PC-tubulin approximately 0.05 mg/mL (Figure 5).

Taxol Does Not Prevent Disassembly of Aspergillus nidulans Microtubules by Cold or Salt but Partially Inhibits Ca^{2+}-Stimulated Disassembly. Taxol stabilizes microtubules of many organisms such that they will not disassemble under several sets of conditions that normally cause microtubule disassembly. To determine if *A. nidulans* microtubules were

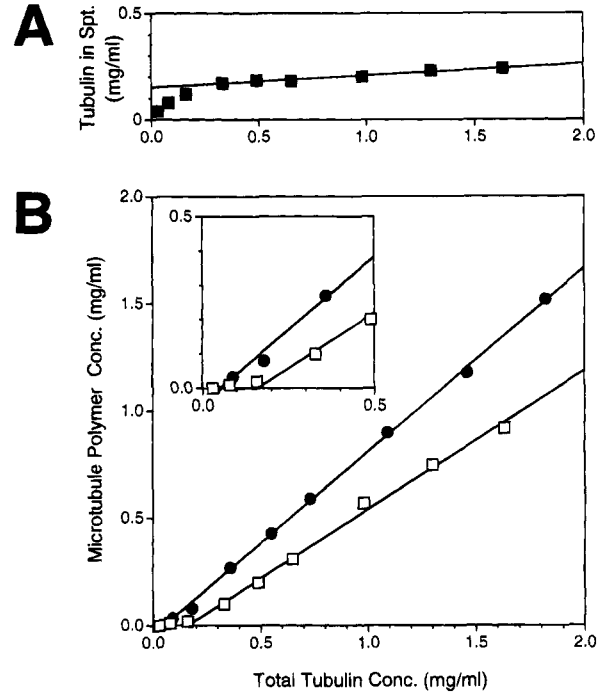

FIGURE 5: Determination of the critical concentration for microtubule assembly in the presence of taxol. Various concentrations of *A. nidulans* tubulin and brain PC-tubulin were incubated (32 °C for *A. nidulans* tubulin and 37 °C for brain PC-tubulin) for 30 min in the presence of 100 and 20 μM taxol, respectively. The *x*-axes for panels A and B are the same and are the total tubulin concentrations (in mg/mL) in the assembly mixtures. Panel A shows the concentration of tubulin in the supernatant after *A. nidulans* tubulin was assembled into microtubules and the microtubules pelleted by centrifugation. At concentrations in which microtubules assemble, the concentration of tubulin in the supernatant should be constant and should correspond to the critical concentration for assembly. At lower concentrations, no assembly will occur, and the supernatant concentration should correspond to the total tubulin concentration. The *y*-intercept of a line plotted through the supernatants from the higher tubulin concentrations gives the critical concentration for assembly (about 0.16 mg/mL). In panel B, the *y*-axis is the concentration of cold-reversible microtubule polymer (the amount of cold-reversible microtubule polymer divided by the volume of the polymerization mixture). This value reflects the proportion of the tubulin that assembles into microtubules. By quantifying cold-reversible polymer rather than total polymer, we eliminate inaccuracies due to denatured tubulin that may sediment but will not subsequently depolymerize. Closed circles denote the values for brain tubulin, and open squares denote the values for *A. nidulans* tubulin. The *x*-intercept (shown at higher magnification in the insert) is the critical concentration for assembly below which no microtubule assembly occurs (about 0.16 mg/mL for *A. nidulans* tubulin and 0.05 mg/mL for brain tubulin). The concentration of cold-reversible polymer was determined as follows. Microtubules were pelleted by centrifugation, and the pellets were depolymerized in the cold in the presence of 3 mM $CaCl_2$ for 45 min. The mixtures were then centrifuged in the cold to remove any denatured tubulin aggregates. Protein concentrations of the supernatants were measured, and the total amount of cold-reversible microtubule polymer was calculated. As mentioned, *y*-axis values were calculated by dividing total cold-reversible polymer by the volume of the assembly mixture.

similarly stabilized, we polymerized *A. nidulans* tubulin into microtubules in the presence of taxol and examined disassembly under various conditions.

Sea urchin microtubules that are assembled in the presence of taxol are cold-stable even after taxol is removed from the solution (Collins & Vallee, 1987). Figure 6A, however, shows that *A. nidulans* microtubules assembled in 100 μM

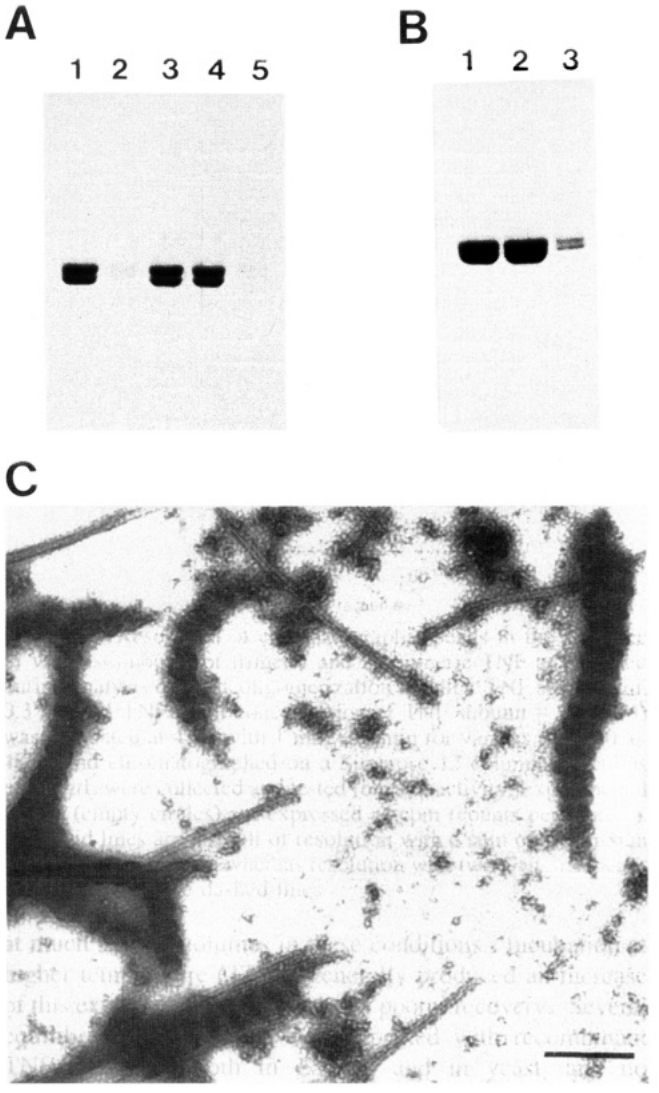

FIGURE 6: Depolymerization of taxol-induced *A. nidulans* microtubules. (A) Coomassie blue-stained gel of microtubule disassembly in the cold in the absence of Ca^{2+}. Microtubules were assembled in the presence of 100 μM taxol (lane 1) and spun down (supernatant, lane 2; pellet, lane 3). The microtubule pellet (lane 3) was resuspended in PEM without Ca^{2+} or taxol. After incubation on ice for 45 min, the material was centrifuged at 4 °C (supernatant, lane 4; pellet, lane 5). (B) Coomassie blue-stained gel of microtubule disassembly in salt. Taxol (100 μM)-assembled microtubules (lane 1) were made to 0.35 M NaCl and incubated at 32 °C for 20 min, and the material was centrifuged (supernatant, lane 2; pellet, lane 3). (C) Electron micrograph showing coiled protofilaments and partially disassembled microtubules caused by Ca^{2+} (scale bar: 0.4 μm). Microtubules were assembled with 100 μM taxol, and $CaCl_2$ was added to 4 mM. The solution was incubated at 32 °C for 90 min before this sample was taken.

taxol, pelleted and resuspended in PEM buffer without taxol, disassemble in the cold even in the absence of Ca^{2+}. We also assembled *A. nidulans* microtubules in 100 μM taxol, placed them on ice (without removing the taxol and in the absence of Ca^{2+}), took samples at time intervals, and examined them by electron microscopy. Microtubules were largely depolymerized after 15 min and were completely undetectable after 75 min (results not shown).

Taxol-treated brain microtubules are stable in 0.35 M NaCl (Vallee, 1982). *A. nidulans* microtubules were assembled with 100 μM taxol, and NaCl was added to a concentration of 0.35 M. The mixture was incubated at 32 °C for 20 min and was then examined by electron microscopy. All microtubules had disappeared, and only a small number of coiled protofilaments remained (results not shown). Centrifugation of the mixture confirmed that the microtubules had largely depolymerized (Figure 6B).

We also tested the sensitivity of taxol-induced *A. nidulans* microtubules to Ca^{2+}. *A. nidulans* microtubules were assembled with 100 μM taxol, and the solution was then made to 4 mM $CaCl_2$. After 15 min at 32 °C, most microtubules were intact. Some coiled protofilament aggregates, however, had appeared. Sixty and ninety minute incubations showed an obvious reduction of the number of microtubules and increase in protofilament aggregates (Figure 6C), but a substantial number of microtubules still remained even after 2 h in 4 mM Ca^{2+}. We obtained quite different results when both taxol and Ca^{2+} were added to *A. nidulans* tubulin in the cold and the mixture was warmed to assemble microtubules. *A. nidulans* microtubule assembly was almost completely inhibited by 4 mM Ca^{2+} even in the presence of taxol. Microtubules were rarely found, and tubulin formed large aggregates of coiled protofilaments. Under the same conditions, however, Ca^{2+} had no effect on the assembly of brain PC-tubulin into microtubules in the presence of taxol (result not shown).

DISCUSSION

Aspergillus nidulans has been remarkably useful for genetic and molecular genetic studies of tubulins, but the lack of a system for purifying assembly-competent tubulin has prevented biochemical analyses of mutant tubulins. We have now developed a procedure that allows us to purify assembly-competent *A. nidulans* tubulin. The procedure involves overexpression of α- and β-tubulin, partial purification by ion-exchange chromatography, and final purification by cycles of assembly and disassembly. The procedure is reasonably efficient, yielding (after two rounds of assembly and disassembly) 2−3 mg of tubulin from a 4 L culture, which corresponds to about 0.2% of total soluble protein. In comparison, the yield for the published tubulin purification procedure for the yeast *Saccharomyces cerevisiae* is approximately 0.05% of soluble protein (Kilmartin, 1981). A modified tubulin purification procedure for *S. cerevisiae* was published recently (Davis et al., 1993) that yields 5−10 mg of tubulin from a 88 L of yeast culture (1−2 kg wet weight of cells).

The degree of overproduction of tubulin we obtained was small (approximately 2−4-fold), and it may consequently seem surprising that we were only able to obtain assembly-competent tubulin from material in which tubulin was overexpressed. We have observed, however, that when we elute tubulin from DEAE-cellulose columns, the ratio of tubulin to contaminating protein is significantly greater in material from the overexpressing strain than in other material (results not shown). This is probably due, in part, to tubulin overexpression, but it is possible that the synthesis of some proteins is inhibited in our induction conditions, which are somewhat toxic to the hyphae. We suspect that some of the proteins that coelute from DEAE-cellulose columns with tubulin may inhibit microtubule assembly and that the major benefit of tubulin overexpression may be to improve the ratio of tubulin to inhibitors. It is also important to note that nucleic acids may coelute with tubulin from DEAE columns

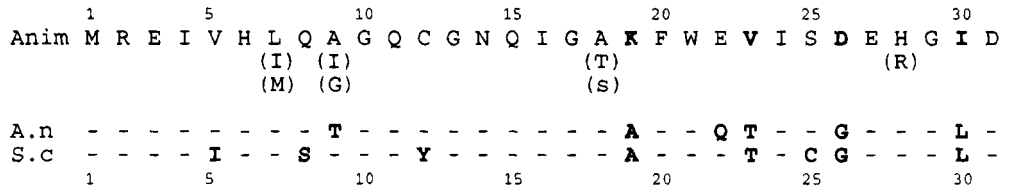

FIGURE 7: Comparison of N-terminal 31 amino acids of β-tubulin from animal (human, pig, chicken, mouse, sea urchin, *Caenorhabditis elegans*, *Tetrahymena pyriformis*, *Drosophila melanogaster*), *Aspergillus nidulans ben*A gene product, and *Saccharomyces cerevisiae* β-tubulin. Sequences were from GenBank release 85, SWISS-PROT release 30, PIR release 42, and PDB release 69.

and inhibit microtubule polymerization (Davis et al., 1993), and it is possible that our inducing conditions might also inhibit nucleic acid synthesis and thus reduce coeluting inhibitors.

The small degree of tubulin overexpression we obtained was surprising in view of the fact that Waring et al. (1989) have found that each copy of *ben*A under control of the *alc*A promoter encodes about 1% of newly synthesized protein after induction for 1.5 h (i.e., the *ben*A, β-tubulin is 1% of total newly synthesized protein when one copy is present, 2% when two copies are present, etc.). We have examined total tubulin, however, instead of newly synthesized tubulin, and it is possible that overexpressed tubulin is subject to proteolysis and does not accumulate to the expected levels.

We were able to obtain consistent assembly of microtubules only in the presence of taxol. Without taxol, *A. nidulans* tubulin assembled predominantly into aggregates of coiled protofilaments reminiscent of vinblastine-induced tubulin aggregates (Erickson, 1975; Warfield & Bouck, 1974; Parness & Horwitz, 1981) and other assembly products formed in the presence of anti-microtubule agents (Mizuno & Suzaki, 1990). These results suggest that the molecular interactions involved in protofilament formation are stronger under our conditions than those that are involved in the assembly of protofilaments into microtubules. They also suggest that the major function of taxol under our conditions may be to facilitate or stabilize interfilament interactions. Since taxol is unlikely to be a natural component of *A. nidulans* hyphae, the failure of microtubule assembly in the absence of taxol may reflect the fact that our assembly conditions are not completely optimal and/or that one or more cellular components important to microtubule assembly (e.g., MAPs) is absent from our preparations (i.e., does not copurify with tubulin on DEAE-cellulose columns). As mentioned, DMSO, glycerol, high Mg^{2+}, and brain MAPs did not promote assembly of microtubules although DMSO did promote protofilament formation. In addition, we used various pHs and different temperatures in an effort to obtain microtubule assembly without taxol but were not successful (results not shown). We also attempted to obtain *A. nidulans* MAPs by mixing tubulin (from brain or from *A. nidulans*) with *A. nidulans* extracts and subjecting the mixture to cycles of microtubule assembly and disassembly. We obtained proteins that copurified with tubulin, but we did not detect any effects of these proteins on microtubule assembly (results not shown).

One of the most interesting results of these experiments is that taxol clearly promotes the assembly of *A. nidulans* microtubules, but higher concentrations of taxol are required to obtain maximal assembly than with mammalian tubulin and the assembled microtubules are less stable to cold, salt, and Ca^{2+} than mammalian microtubules stabilized with taxol. The requirement for high taxol concentrations for *A. nidulans* microtubule assembly could reflect a higher stoichiometry of binding of taxol to *A. nidulans* tubulin than mammalian tubulin or, much more likely, a lower binding affinity to *A. nidulans* tubulin. Even at taxol concentrations that give maximal assembly, *A. nidulans* microtubules are less stable to cold, NaCl, and Ca^{2+} than mammalian microtubules. In comparison, Barnes et al. (1992) reported that taxol has no effect on the assembly of tubulin from the budding yeast *Saccharomyces cerevisiae*. Taxol stabilizes microtubules prepared from plant cells quite efficiently, but the binding affinity of taxol to plant tubulin may be lower than to brain tubulin (Bokros et al., 1993).

The taxol binding site on brain tubulin has recently been narrowed, by cross-linking studies, to the first 31 amino acids of β-tubulin (Rao et al., 1994) (although it is theoretically possible that other regions might sterically affect binding). Figure 7 shows a comparison of this region in animal, *A. nidulans*, and *S. cerevisiae* β-tubulins. While we can obviously draw no firm conclusions about taxol binding from this comparison alone, several points are worth mentioning. First, this region is highly conserved among β-tubulins. A small number of amino acid substitutions probably account for the differences in taxol effects. Second, *A. nidulans* and *S. cerevisiae* β-tubulins are identical to each other but different from animal β-tubulins at amino acids 19, 23, 26, and 30. One or more of these differences may account, at least in part, for the reduced effects of taxol on *A. nidulans* and *S. cerevisiae* tubulins. Third, at four positions, amino acids 5, 8, 12, and 25, *A. nidulans* and animal β-tubulins are identical but *S. cerevisiae* β-tubulin differs. The identity between *A. nidulans* and animal tubulins at one or more of these residues may account for the greater effect of taxol on *A. nidulans* microtubules than on *S. cerevisiae* microtubules. In any case, the ease of molecular genetic manipulations in *A. nidulans*, coupled with the tubulin purification procedure we have developed, raises the exciting possibility of testing the effects of each of these residues on taxol binding and thus of defining the site and nature of taxol binding with great precision.

ACKNOWLEDGMENT

We thank Dr. Matthew Suffness of the National Cancer Institute for the gift of taxol, Dr. Gregory May for pALT6, Dr. Ronald Morris for the *tub*A gene, Dr. Lester Binder for Tu27B, the departments of Plant Biology and Zoology at The Ohio State University for the use of their electron microscope and darkroom facilities, and Dr. M. Katherine Jung, Dr. Tetsuya Horio, and C. Elizabeth Oakley for reading the manuscript.

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BI950015N