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Transfer and Amplification of a Mutant β-Tubulin Gene Results in Colcemid Dependence: Use of the Transformant To Demonstrate Regulation of β-Tubulin Subunit Levels by Protein Degradation

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Total genomic DNA from a temperature-sensitive, colcemid-resistant Chinese hamster ovary (CHO) cell mutant expressing an electrophoretic variant β-tubulin was used to transform wild-type CHO cells to colcemid-resistant cells at 37°C. Southern blot analysis of the transformant demonstrated the three- to fivefold amplification of one of many β-tubulin sequences compared with that of the wild type or mutant, thereby identifying a functional tubulin gene in CHO cells. This amplification of one tubulin-coding sequence resulted in a threefold increase in two β-tubulin mRNA species, suggesting that both species may be encoded by a single gene. Pulse-chase experiments showed that in the transformant, total β-tubulin was synthesized and degraded faster than in the revertant or wild-type cells, so that the steady-state levels of β-tubulin and α-tubulin were unchanged in the transformant compared with those of wild-type, mutant, or revertant cells. Increased ratios of mutant to wild-type β-tubulin made the transformant dependent on microtubule-depolymerizing drugs for growth at 37 but not 34°C and supersensitive to the microtubule-stabilizing drug taxol at 34°C.

Heterodimers of α- and β-tubulin polymerize to form microtubules, which are the main components of mitotic and meiotic spindles, eucaryotic cilia and flagella, and neuronal processes. In addition, cellular microtubules are involved in intracellular transport and saltatory movement of cytoplasmic organelles as well as in membrane function. Mammalian cells have 15 to 20 genes per haploid genome for both α- and β-tubulin (11). Only three human β-tubulin DNA sequences encoding three distinct protein isotypes (21, 22, 29) and two human α-tubulin sequences (15) are known to represent functional genes. Many of the mammalian tubulin sequences are pseudogenes containing genetic lesions which prevent synthesis of a functional protein. In several cases intronless, poly(A)$^+$ pseudogenes are observed (21, 28, 42, 43).

The expression of different tubulin genes is tissue specific and differentially regulated during embryonic development (15, 23, 31). Frequently, more than one β-tubulin gene is expressed in a particular tissue (22, 23). It is unclear if different tubulin genes encode proteins with different functions. Genetic studies with *Drosophila melanogaster* (24, 25) and with Chinese hamster ovary (CHO) cells (7) suggest that mutations in a single gene affect functionally different microtubules. On the other hand, there is evidence that different α-tubulins, distinguishable by their reaction with monoclonal antibodies, are associated with physiologically different microtubules within the same cell (40).

The identification of the expressed tubulin genes and the elucidation of their function and regulation in mammalian cells have been our major goal. To this end, we have isolated several codominant mutants with variant α- and β-tubulins in cultured CHO cells on the basis of resistance of these cells to the microtubule-depolymerizing drugs colcemid (Cmd), colchicine, and griseofulvin (5) or the microtubule-stabilizing drug taxol (4). One Cmd-resistant mutant (Cmd-4) has an electrophoretically variant β-tubulin in addition to the normal protein; the altered β-tubulin is not the result of a posttranslational modification (8). Since the ratio of the newly synthesized mutant to wild-type β-tubulin protein was 1:2, we concluded that there are probably three functional β-tubulin genes per diploid CHO genome. The Cmd-4 mutation results in a temperature-sensitive phenotype because of a defect in the formation of mitotic spindles at the nonpermissive temperature; however, cellular microtubules are not affected in this mutant (1, 5). The presence of an isoelectric variant tubulin in Cmd-4 provides a biochemical marker for the altered gene and has allowed us to follow the functional and regulatory consequences of transferring and amplifying this gene.

In this work, we demonstrate that a gene encoding this mutant β-tubulin can be transferred from the original mutant CHO cell line to a wild-type CHO cell. Amplification of the mutant gene in the transformant cells results in increased expression of two β-tubulin mRNA species and increased ratios of mutant to wild-type β-tubulin, resulting in a Cmd-dependent phenotype of the transformant cells.

MATERIALS AND METHODS

Cell lines and plasmids. The wild-type CHO cells, β-tubulin mutant (Cmd-4), and medium (α-modified Eagle) were described previously (2, 8). The transformant was maintained in medium containing 30 ng of Cmd per ml. pSV2-*neo* plasmid (38) was kindly provided by Bruce Howard (National Institutes of Health), and the human β-tubulin plasmid (pDβ-1) isolated in the laboratory of N. Cowan (New York University School of Medicine) (22) was from Don Cleveland (Johns Hopkins University School of Medicine). The CHO α-tubulin clone cDNA clone was isolated in this laboratory by C. Whitfield and P. Doherty from a CHO cDNA library prepared by the method of Okayama and Berg (34). The CHO α-tubulin was identified by colony hybridization (20) and Southern blotting (described below) with nick-translated, ^{32}P-labeled chicken α-tubulin (11) as a probe. The chicken α-tubulin and CHO α-tubulin plasmids hybrid selected the

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same CHO mRNA, resulting in the same size protein after in vitro translation (11).

Plasmids were purified by double banding in a CsCl-ethidium bromide gradient (18). After digestion of pDβ-1 with *Pst*I (New England BioLabs, Inc.) and electrophoresis in a 0.9% low-melting-temperature agarose gel, the human β-tubulin insert was cut out of the agarose gel, heated, and purified with an Elutip, as described by Schleicher & Schuell, Inc. The CHO α-tubulin plasmid was digested with *Pst*I and *Hin*dIII, and the 1,100-base-pair insert was electroeluted from the gel (32) and purified with an Elutip.

The α- and β-tubulin inserts were ^{32}P labeled by nick translation and purified by spermine precipitation (19). The nick translation conditions of Rigby et al. (36) were modified as follows: the reaction mixture contained 5 mM Tris chloride (pH 7.4); 5 mM $MgCl_2$; 10 mM β-mercaptoethanol; 0.5 mg of bovine serum albumin per ml; 2 μM dATP, dTTP, dGTP, and dCTP; 800 μCi of [α-^{32}P]dGTP per ml; 800 μCi of [α-^{32}P]dCTP per ml; 50 mU of DNase I per ml (Boehringer Mannheim Biochemicals); 50 U of DNA polymerase (Kornberg; Boehringer Mannheim); and 10 μg of DNA per ml. Incubation was at 14°C for 20 to 25 min.

Transfer of Cmd resistance. Wild-type CHO cells were treated for 20 h with a $CaPO_4$ precipitate of purified β-tubulin mutant or wild-type genomic Chinese hamster DNA (10 μg/100-mm dish) and purified pSV2-*neo* plasmid DNA (10 μg/100-mm dish) as described by Abraham et al. (2). Twenty-four hours after replacement of the $CaPO_4$ precipitate with normal medium, cells were replated at 5×10^5 cells per 100-mm dish in medium plus 1.6 mg of the neomycin analog G418 (GIBCO Laboratories) per ml (38). After 65 h, medium was changed to Cmd (0.04 μg/ml) medium. Colonies were picked into Cmd medium after 18 days.

DNA analysis. High-molecular-weight genomic DNA was isolated from CHO cells (2) and digested with *Hin*dIII or *Bam*HI according to the directions of the distributor (New England BioLabs). The digested DNA was electrophoresed in a 0.9% agarose gel with Tris acetate (40 mM)–EDTA (2 mM) buffer. After treating with 0.25 M HCl for 10 min, we prepared the gel by the method of Southern (37) and then soaked it in 10× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaP_i [pH 7.4], 1 mM EDTA). DNA was blotted to Gene Screen (New England Nuclear Corp.) with 2× SSPE; the Southern blot was rinsed briefly with 2× SSPE, baked at 80°C for 2 h, and stored frozen. The Gene Screen was prehybridized with 10× Denhardt solution (10× Denhardt solution is 0.2% Ficoll [Pharmacia Fine Chemicals], 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), 50% formamide (Fluka), 10% dextran sulfate (Pharmacia), 5× SSPE, 1% sodium dodecyl sulfate (SDS), and 100 μg of *Micrococcus lysodeikticus* DNA per ml (Sigma Chemical Co.; type X1) for 2 to 4 h at 42°C; then a heat-denatured (5 min at 100°C), nick-translated, ^{32}P-labeled human β-tubulin or CHO α-tubulin probe was added, and hybridization was carried out for 16 to 18 h at 42°C (19). The blot was washed three times at room temperature with 2× SSPE–0.1% SDS for a total of 30 to 60 min; in some cases the blot was washed twice at 60°C with 2× SSPE–1% SDS for a total of 60 min; and finally it was washed three times at 52°C with 0.1× SSPE–0.1% SDS for a total of 45 to 60 min. The filter was exposed to prefogged X-ray film, and bands were quantitated with a 3CS densitometer (Joyce-Loebel).

RNA analysis. RNA was prepared from CHO cells by the guanidinium-CsCl method (17, 41) as modified by Doherty et al. (16). For RNA slot blots, total RNA diluted with 1× SSPE in 200 μl was applied to the Gene Screen (presoaked in 1× SSPE) with a Minifold two-slot blotter (Schleicher & Schuell) and allowed to sit for 2 h or longer without suction. For Northern blots, total denatured RNA was electrophoresed in formaldehyde-agarose gels and transferred to the Gene Screen as described by Graham et al. (19). After baking at 80°C for 2 h, RNA blots were hybridized to ^{32}P-labeled human β-tubulin as described for DNA blots, omitting the wash at 60°C with 2× SSPE–1% SDS. Filters were exposed to X-ray film, and bands were quantitated with a densitometer (Joyce-Loebel). Blots were washed as described by Graham et al. (19), or the radioactivity was allowed to decay before hybridization to ^{32}P-labeled CHO α-tubulin cDNA. The α-tubulin probe was prehybridized to the Gene Screen and then redenatured at 80°C for 10 min; the hybridization conditions for DNA blots were used except that Northern blots were washed at 61°C with 0.1× SSPE.

Protein analysis. Two-dimensional gel electrophoresis and [^{35}S]methionine labeling was as previously described (6) except that methionine-free medium was used for labeling. In some experiments where β-tubulin spots were excised from two-dimensional gels, partially purified microtubule-containing extracts were prepared by treating cells with 0.05% Nonidet P-40–0.01 M Tris (pH 7.4) and acetone-precipitating [^{35}S]methionine-labeled protein in the supernatants before two-dimensional electrophoresis. In these experiments, the method of O'Farrell (33) was used for two-dimensional analysis. This partial purification excludes many insoluble proteins such as vimentin, which frequently comigrates with α-tubulin from the extracts (8, 9). The excised gel pieces were treated with 1 ml of 30% H_2O_2–1% NH_4OH for 18 h at 37°C and counted with 15 ml of Biofluor scintillant in a liquid scintillation counter (Beckman Instruments, Inc.).

Measurement of cell growth. For high-density growth curves, 10^4 cells were plated per 30-mm dish or per 16-mm well of a 24-well dish in medium with or without 0.03 μg of Cmd per ml. At various times cells from duplicate dishes were removed with 0.25% trypsin–0.7 mM EDTA and counted with a Coulter Counter (Coulter Electronics, Inc.). Low-density growth in two to four replicate samples was measured by plating 200 cells per 60-mm dish or per 16-mm well of a 24-well dish. Drug resistance curves were obtained by seeding 200 cells per 16-mm well of a 24-well dish. After optimal time for colony formation in controls (about 10 days at 34°C, 7 days at 37°C, or 9 days at 40.5°C), colonies were stained with 0.5% methylene blue in 50% ethanol and were counted. The concentration of antimitotic drug decreasing the maximum plating efficiency by 50% (LD_{50}) was calculated from the killing curves. Relative plating in antimitotic drugs was compared by dividing the LD_{50} of mutant, transformant, or revertant cells by that of wild-type cells. Reproducible results were obtained for relative resistance determined two to four times for antimitotic drugs (except that podophyllotoxin was measured only once).

RESULTS

DNA-mediated transfer of Cmd resistance. The codominant Cmd-resistant phenotype was transferred with genomic DNA from the β-tubulin mutant Cmd-4 mixed with pSV2-*neo* plasmid DNA. Transformants were selected in two steps. (i) Cells were selected with the neomycin analog G418 for several days to enrich for cells that had taken up the transferred DNA, thereby reducing the background of spontaneous Cmd resistance. (ii) Cells surviving the G418 selection were selected for resistance to Cmd. The overall frequency of Cmd resistance with this double selection was

about 3 in 10^7 cells. Cmd-resistant clones were also found at a similar frequency on the control plates in which wild-type genomic DNA was used. These control clones were also puromycin resistant, however, an indication that they expressed the multidrug-resistant phenotype, which is unrelated to alterations in tubulin and arises very frequently in CHO cells (8, 30).

One cell line arising from the cotransfer of mutant DNA and the pSV2-*neo* plasmid exhibited the properties expected for a transformant. This cell line (11801) was Cmd resistant and puromycin sensitive to the same extent as the original Cmd-4 mutant was. As expected, this transformant was resistant to G418, indicating that the transformant had acquired the cotransferred DNA.

Amplification of a β-tubulin gene. After digestion with restriction enzymes, genomic DNA from the putative transformant was analyzed by electrophoresis and Southern blotting in expectation of a new or amplified band hybridizing to the human β-tubulin probe. Multiple bands were observed in Southern blots of the DNA from CHO cells with the human β-tubulin probe, suggesting the presence of multiple tubulin genes (Fig. 1). The data from this experiment were quantitated by densitometry of the autoradiograms and normalized to an unaffected band. A single *Hin*dIII restriction fragment of approximately 13 kilobases (kb) was amplified approximately two- to fourfold in the transformant compared with the wild type or Cmd-4 mutant (Fig. 1A and data not shown). With *Bam*HI, an approximately 6-kb fragment was amplified four- to fivefold in the transformant compared with the wild type, Cmd-4 mutant, or a control cell line which had been selected in Cmd after treatment with wild-type DNA (Fig. 1B). In one experiment (Fig. 1), *Bam*HI digestion of the transformant and control was not quite complete; however, the degree of digestion did not affect the amount of the amplified fragment or the 15-kb fragment used for normalization, since the same ratio (6 kb/15 kb peak measured after densitometry) was observed in the incompletely digested control as in the completely digested wild type or Cmd-4 mutant. The amplified β-tubulin gene in the transformant was not caused by an increased number of chromosomes. The metaphase modal chromosome number of the transformant was 19, and that of the wild-type recipient was 20 to 21.

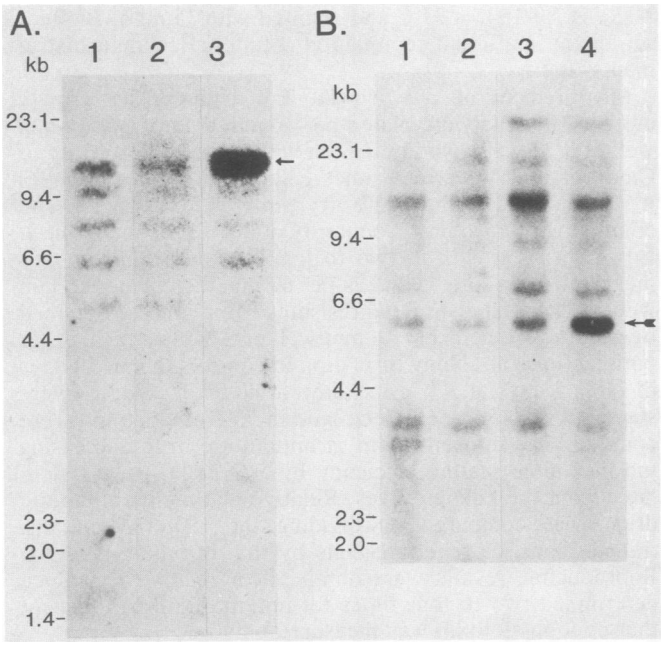

FIG. 1. Southern blot of restriction-endonuclease-digested genomic CHO DNA hybridized with human β-tubulin cDNA. (A) *Hin*dIII-digested DNA. A sample (16 μg) was electrophoresed in 0.9% agarose, blotted onto Gene Screen, probed with ^{32}P-labeled human β-tubulin cDNA, and autoradiographed. Lanes: 1, wild type; 2, Cmd-4 mutant; 3, transformant selected in Cmd after treatment of wild-type cells with Cmd-4 mutant DNA. (B) *Bam*HI-digested DNA. A sample (15 μg) was electrophoresed, transferred to Gene Screen, and hybridized with ^{32}P-labeled human β-tubulin cDNA. Lanes: 1, wild type; 2, Cmd-4 mutant; 3, control cell line selected in Cmd after treatment of wild-type cells with wild-type DNA; 4, transformant. The arrows point to the restriction fragment which is amplified in the transformant.

When genomic DNA was restricted, electrophoresed, blotted, and hybridized to a ^{32}P-labeled CHO α-tubulin cDNA, no differences in the multiple α-tubulin bands were observed in the transformant compared with the blots of mutant or wild type (data not shown).

Amplification of β-tubulin RNA. To determine if β-tubulin mRNA was amplified in the transformant, we applied the total RNA directly to Gene Screen on a slot blot apparatus or transferred it to Gene Screen after electrophoresis in formaldehyde-agarose gels (Northern blotting) and probed with labeled human β-tubulin cDNA. In RNA slot blots (Fig. 2A), densitometric analysis revealed a threefold amplification of β-tubulin mRNA in the transformant compared with the wild type or β-tubulin mutant. Furthermore, both species of β-tubulin mRNA (2.2 and 3.2 kb; Fig. 2B) normally observed in CHO cells (12) were amplified approximately threefold as measured by densitometry. As a control for the amount of total RNA present, the same RNA slot blot (Fig. 2A) and Northern blot (Fig. 2B) were hybridized with ^{32}P-labeled CHO α-tubulin cDNA after the β-tubulin probe was washed off. No differences were detected with the α-tubulin probe in the transformant compared with the wild type or mutant (Fig. 2C and D).

Mutant β-tubulin protein in the transformant. Two-dimensional gel electrophoresis was conducted to detect the electrophoretic variant β-tubulin in the mutant and transformant. In the Cmd-4 mutant, both normal β-tubulin and a mutant protein with a more basic isoelectric point were observed on two-dimensional gels (8). The identity of the mutant β-tubulin was verified by copurification with normal β-tubulin and by protease maps (one-dimensional V8 protease maps and two-dimensional tryptic peptide maps) similar to those of wild-type β-tubulin (8). A new spot, having exactly the same mobility as the mutant β-tubulin in Cmd-4, was seen on two-dimensional gels of the Cmd-resistant transformant (Fig. 3C). In addition, the mutant β-tubulin was not detected in a Cmd-sensitive revertant, selected by growing the transformant in medium without Cmd for several weeks at 37°C (Fig. 3D) (see below). The ratio of mutant to wild-type β-tubulin in Cmd-4 was approximately 1:2 (8), while the ratio in the transformant was at least 1:1 (Fig. 3C). The higher ratio of mutant to wild-type β-tubulin in the transformant strongly suggests that the amplification of a specific β-tubulin gene in the transformant is due to the transfer and amplification of the Cmd-4 mutant β-tubulin gene and not to a spontaneous mutation. It is unlikely that a spontaneous mutation would have resulted in the same alteration in tubulin protein structure as that represented by the altered spot seen in the Cmd-4 mutant.

Steady-state levels of α- and β-tubulin. The steady-state

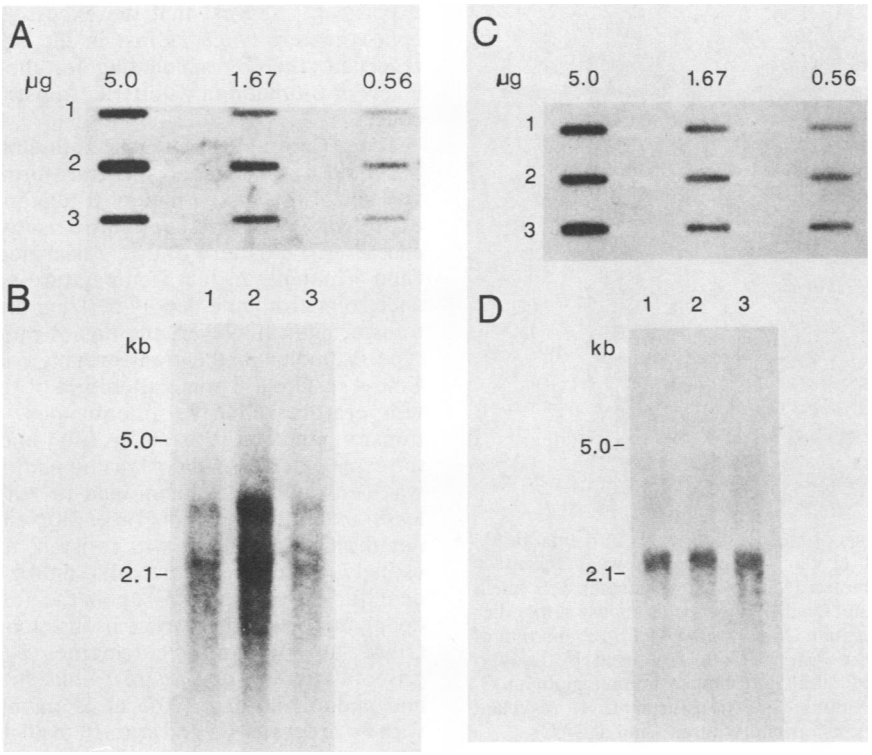

FIG. 2. Blot analysis of CHO tubulin RNA. (A) Slot blot analysis. Total RNA, 0.56 to 5 μg as determined by UV absorption, was applied to Gene Screen with a slot-blotting apparatus, hybridized to ^{32}P-labeled human β-tubulin cDNA, and autoradiographed. Lanes: 1, wild type; 2, transformant; 3, Cmd-4 mutant. (B) Northern blot analysis. Total RNA, 21 μg, was denatured and electrophoresed in 1.4% formaldehyde–agarose gels, transferred to Gene Screen, hybridized to ^{32}P-labeled human β-tubulin cDNA, and autoradiographed. Lanes: 1, wild type; 2, transformant; 3, Cmd-4 mutant. rRNA bands stained with ethidium bromide appeared to be the same intensity in different lanes. The size of the CHO β-tubulin mRNAs was calculated from the ribosomal markers at 5.0 and 2.1 kb (27). (C) Same slot blot as in panel A hybridized to a ^{32}P-labeled CHO α-tubulin probe after radioactive decay of the β-tubulin probe. (D) Same Northern blot as in panel B hybridized to ^{32}P-labeled CHO α-tubulin probe after washing to remove the β-tubulin probe. In both slot blots and Northern blots, replicate samples gave identical results.

levels of α- or β-tubulin were quantitatively compared in different cell lines by applying equal amounts of total protein to nitrocellulose with a slot blotter and then by inducing reaction with monoclonal, monospecific antibodies to α- or β-tubulin. The level of β-tubulin was not different in wild-type, mutant, transformant, or revertant cell lines; likewise, the α-tubulin level was approximately the same in these cell lines (Fig. 4).

Pulse-chase experiments with [^{35}S]methionine. When proteins are pulsed for short periods with [^{35}S]methionine and chased with cold methionine, the labeling observed is primarily a function of the rate of protein synthesis in the pulse and a measure of protein degradation during the chase. In several experiments, growing transformant, revertant, and wild-type cells were pulse labeled for 30 min and chased for 14, 18, or 24 h. Either total cellular proteins were extracted in a 9.5 M urea buffer or partially purified microtubule proteins were fractionated on two-dimensional gels, and the

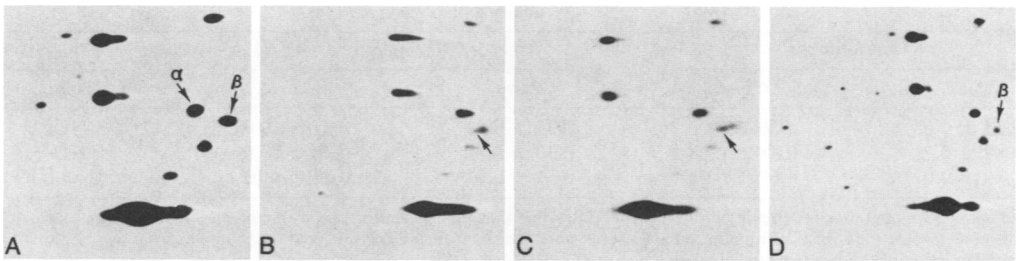

FIG. 3. Two-dimensional gel autoradiograms of wild type (A), Cmd-4 mutant (B), transformant (C), and revertant (D). Cells were pulse-labeled with [^{35}S]methionine for 2 h and lysed with SDS. Proteins were separated by isoelectric focusing (across, basic on left), then by SDS-gel electrophoresis (down). Only a portion of the gel is shown. α, α-Tubulin; β, wild-type β-tubulin. Arrows in panels B and C point to the isoelectric variant, mutant β-tubulin. The revertant (D) was selected by growing the transformant cells in medium without Cmd at 37°C for several weeks.

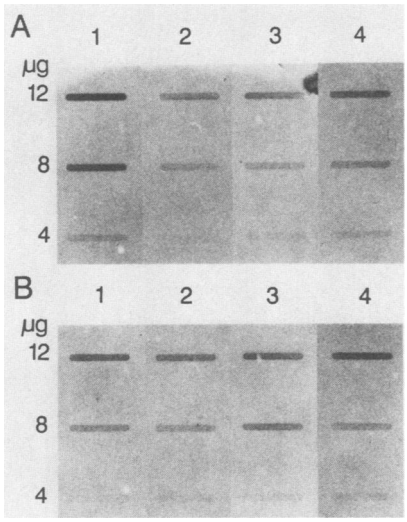

FIG. 4. Slot blot analysis of steady-state levels of α-tubulin (A) and β-tubulin (B). Total protein (4 to 12 μg) was applied to nitrocellulose with a slot blotter (Minifold 2, Schleicher & Schuell) and then reacted with monoclonal, monospecific mouse antibodies to chicken α-tubulin (Amersham Corp.) or to CHO β-tubulin (gift of L. I. Binder, University of Virginia) (26), followed by affinity-purified peroxidase-labeled rabbit anti-mouse immunoglobulin G. Lanes: 1, wild type; 2, mutant; 3, transformant; 4, revertant (selected by growing the transformant without Cmd at 37°C).

radioactivity in the β-tubulin spots and several control spots including the actin spot was measured. The ratio of the radioactivity in β-tubulin to that of actin was used as a measure of the relative synthesis or degradation rate of β-tubulin. During the pulse, the ratio of radioactivity in β-tubulin to that of actin was three- to sixfold higher in the transformant than in revertant or wild-type cells, indicating that the rate of synthesis of β-tubulin was greater in the transformant cells (Table 1). No similar changes in ratios between a randomly chosen radioactive spot and an actin spot were seen in the transformant relative to the ratios of revertant or wild-type cells (data not shown). After the 14-, 18-, or 24-h chase, the ratios of β-tubulin to actin in the transformant decreased 1.5- to 2-fold and thus were closer to the ratios in the revertant or wild-type cells (Table 1). On the other hand, the ratios of β-tubulin to actin in the revertant were the same in the pulse as in the chase. These chase experiments suggest that the excess β-tubulin is degraded approximately twice as fast in the transformant as in the revertant, thereby accounting for the similar steady-state levels of β-tubulin in wild-type, transformant, and revertant cells.

Temperature sensitivity and antimitotic drug resistance. If the Cmd resistance of the transformant is due to gene transfer of the Cmd-4 mutant β-tubulin, the transformant is expected to exhibit temperature sensitivity at 40.5°C and the characteristic pattern of drug resistance associated with the Cmd-4 mutant. In fact, temperature sensitivity and resistance to microtubule-depolymerizing drugs were seen in the transformant; however, the higher ratio of mutant to wild-type β-tubulin in the transformant compared with that in Cmd-4 resulted in some alteration of the drug-resistant and temperature-sensitive phenotypes. For example, the transformant was resistant to Cmd and podophyllotoxin to the same extent as Cmd-4 was; in addition, the transformant was resistant to colchicine and to vinblastine to a slightly lesser extent than Cmd-4 (Table 2). Neither the transformant nor the Cmd-4 mutant was resistant to griseofulvin. Interestingly, the transformant cells, unlike Cmd-4, were dependent for growth at 37°C upon the following microtubule-depolymerizing drugs with various cloning efficiencies: Cmd (100% at 30 ng/ml), colchicine (104% at 100 ng/ml), griseofulvin (89% at 4 μg/ml), vinblastine (100% at 5 ng/ml), and podophyllotoxin (70% at 15 ng/ml); cloning efficiency with no drug was 4% and with 10 to 80 ng of taxol per ml was 0%.

At 34°C the transformant was able to grow without microtubule-depolymerizing drugs (Table 3, Fig. 5). On the other hand, at 34°C the transformant was 10 times more sensitive to the microtubule-stabilizing drug taxol than Cmd-4 was, which was also somewhat more sensitive to taxol than the wild type was at 34°C (Table 2).

At 40.5°C the transformant was temperature sensitive for growth with or without Cmd (Table 3, Fig. 5). As seen in the transformant at 37°C, Cmd-4 cells were dependent on Cmd for growth to a high density at 40.5°C (Fig. 5).

A revertant, selected at 37°C by growing the transformant in medium without Cmd for several weeks, exhibited approximately the same sensitivity to temperature and Cmd or podophyllotoxin as the wild type did (Tables 2 and 3). As noted above, this revertant has lost the mutant β-tubulin spot (Fig. 3D), characteristic of the Cmd-4 mutant and transformant (Fig. 3B and C), and has served as an important control in many of our experiments.

TABLE 1. Synthesis and degradation of β-tubulin in transformant and other cells

Expt	β-Tubulin/actin (cpm/cpm) ± SD					
	Transformant		Revertant		Wild type	
	Pulse	Chase	Pulse	Chase	Pulse	Chase
1[a]	0.13 ± 0.05	.058 ± 0.02	.033 ± 0.02	.037 ± 0.02	ND[b]	ND
2[c]	0.89 ± 0.21	0.58 ± 0.08	0.30 ± 0.08	0.28 ± 0.07	ND	ND
3[d]	1.26 ± 0.01	0.67 ± 0.02	0.31 ± 0.03	0.28 ± 0.02	0.21 ± 0.09	ND

[a] Cells were pulsed with [^{35}S]methionine for 30 min or pulsed with [^{35}S]methionine and chased for 18 h with cold methionine. After extraction with urea sample buffer and two-dimensional gel electrophoresis, β-tubulin and actin spots were excised from the gels and counted. Samples from three different gels were obtained; the mean and standard deviation of the ratios were calculated.

[b] ND, Not determined.

[c] Cells were pulsed with [^{35}S]methionine for 30 min or pulsed with [^{35}S]methionine and chased for 14 h with cold methionine. Partially purified microtubule-containing extracts were prepared by extracting cells with 0.05% Nonidet P-40–0.01 M Tris chloride (pH 7.4), and aliquots were acetone precipitated before dissolving in urea sample buffer. β-Tubulin, actin, and an unknown protein were excised from two-dimensional gels and counted. Triplicate samples were obtained and calculated as in experiment 1.

[d] Cells were pulsed and extracted as in experiment 2, except that the chase was for 24 h. Duplicate samples were obtained, and the means are given.

TABLE 2. Relative LD_{50} of antimitotic drugs for various cell lines compared with that for the wild type

Cell line	LD_{50}[a]					
	Cmd	Colchicine	Podophyllotoxin	Vinblastine	Griseofulvin	Taxol
Wild type[b]	1	1	1	1	1	1
Cmd-4	3.2	3.3	3.4	2.7	1.1	0.56
Transformant	3.7	1.8	3.3	1.9	0.9	0.059
Revertant	1.5	NT[c]	0.9	NT	NT	NT

[a] Relative LD_{50} (concentration of drug which decreases the maximum plating efficiency by 50%) was compared by dividing the LD_{50} of mutant, transformant, or revertant cells by that of wild-type cells. Growth temperature was 37°C with all drugs except vinblastine and taxol (both at 34°C).

[b] LD_{50}s for wild-type cells: 14 ng/ml (Cmd), 0.11 μg/ml (colchicine), 6.5 ng/ml (podophyllotoxin), 16 ng/ml (vinblastine), 6.7 μg/ml (griseofulvin), and 98 ng/ml (taxol).

[c] NT, Not tested.

DISCUSSION

The transfer of the mutant β-tubulin gene into wild-type CHO cells was undertaken to demonstrate the linkage of the Cmd-resistant phenotype to the mutant β-tubulin protein and to place the mutant gene into an unmutagenized genetic background. The key ingredient in the successful transfer of the mutant β-tubulin gene was the double selection, first with the neomycin analog G418 to enrich for cells which had taken up the pSV2-*neo* plasmid, and then with Cmd. Of the multiple β-tubulin restriction fragments observed on Southern blotting of genomic CHO DNA, only one was amplified three- to fivefold in the transformant, thereby identifying a functional β-tubulin gene in CHO cells and assigning the original mutation to a specific restriction fragment. No alterations in the pattern of restriction fragments hybridizing to a β-tubulin probe have ever been observed with any of our other tubulin mutants (Abraham and Gottesman, unpublished data) or with the controls that have wild-type DNA in the DNA transfer (Fig. 1); therefore, it is unlikely that the amplification is the result of a spontaneous mutation. It is likely that amplification occurs after gene transfer, since the transformant was not as Cmd dependent in initial studies as in later experiments. Nevertheless, DNA-mediated transfer of a mutant gene is a unique way to identify a functional gene in a multigene family. The construction of this amplified transformant will facilitate the cloning of the mutant gene.

The amplification of a single β-tubulin coding sequence resulted in a threefold increase of the two species of β-tubulin mRNA (2.2 and 3.2 kb) normally found in CHO cells (12), suggesting that both of these species may be encoded by a single gene. We cannot rule out the possibility that two linked, expressed β-tubulin genes were transferred. This seems unlikely since the mutant β-tubulin was overexpressed relative to the wild-type β-tubulin subunit in the transformant (see below). In addition, in human cells two RNA species (1.8 and 2.6 kb) are transcribed from the same β-tubulin gene due to alternative polyadenylation sites (21). No differences in levels of α-tubulin mRNA were detected in the transformant.

A new peptide with precisely the same mobility as the Cmd-4 mutant β-tubulin was observed on two-dimensional gel electrophoresis of the Cmd-resistant transformant; this isoelectric variant was uncommon (1 in 40) among mutants resistant to Cmd, colchicine, and griseofulvin (8). Thus, it is exceedingly unlikely that a spontaneous Cmd-resistant mutant with the same isoelectric variant β-tubulin that Cmd-4 has would be detected among the neomycin-resistant colonies. Furthermore, the ratio of mutant to wild-type β-tubulin in the transformant was greater than in Cmd-4, as might be expected if the mutant gene was amplified and overexpressed in the transformant compared with Cmd-4. Taken together, these data demonstrate that the Cmd resistance of the transformant is due to transfer and amplification of the Cmd-4 mutant β-tubulin gene.

Pulse-chase experiments demonstrated that β-tubulin protein is both synthesized faster (at least threefold) and degraded faster in the transformant than in the revertant or wild-type cells. Since Spiegelman et al. (39) report a half-life of approximately 48 h for tubulin peptides from wild-type CHO cells, the half-life for β-tubulin appears to be approximately 16 to 24 h in the transformant. As a result of the more rapid β-tubulin protein degradation in the transformant, the steady-state levels of β- and α-tubulin remain unchanged in the transformant compared with those of mutant, wild-type, or revertant cells. A similar example of regulation of levels of unpolymerized spectrin monomer by protein degradation has recently been reported (44).

These observations have implications for the regulation of tubulin expression. In CHO and other eucaryotic cells, the rates of α- and β-tubulin protein synthesis are thought to be controlled by an autoregulatory mechanism involving the level of unpolymerized αβ-tubulin dimers. For example, new synthesis of α- and β-tubulin is repressed by elevating the level of unpolymerized tubulin dimers with the microtubule-depolymerizing drugs colchicine or nocodazole (3, 12) or with microinjection of purified depolymerized α- and β-tubulin subunits (13). Repression of new tubulin synthesis by tubulin subunits is mediated by a rapid decrease (half-time, 1 h) in cytoplasmic tubulin mRNA steady-state levels (10, 12). The decrease in tubulin mRNA levels is probably not controlled at the level of transcription, since tubulin RNA synthesis in vitro that is measured in nuclear runoff experiments with isolated nuclei from colchicine-treated or from control cells is the same (10). The mechanism of autoregu-

TABLE 3. Cloning efficiency of various cells with and without Cmd at different temperatures

Cell line	Cmd	% Cloning efficiency[a] at °C:		
		34	37	40.5
Wild type	−	77	100	68
Wild type	+	2	0	0
Cmd-4	−	111	100	0
Cmd-4	+	98	92	0[b]
Transformant	−	72	6	0
Transformant	+	102	100	0
Revertant	−	84	100	93
Revertant	+	1	0	NT[c]

[a] 100% cloning efficiency is the number of colonies obtained at 37°C without Cmd for the wild type, mutant, and revertant and the number of colonies at 37°C with Cmd (30 ng/ml) for the transformant.

[b] Occasionally a few colonies were observed.

[c] NT, Not tested.

lation has not been elucidated; perhaps, regulation of tubulin mRNA is at the level of RNA processing, transport, or degradation (14).

Our data are consistent with the autoregulatory model and, in addition, support a role for protein degradation in regulating tubulin levels. The elevated levels of β-tubulin RNA in the transformant are the result of an increased number of functional β-tubulin genes. As predicted by the autoregulatory model, the β-tubulin RNA levels remain high in the transformant because the steady-state concentration of αβ-tubulin dimer is unchanged compared with that of the wild type. The faster degradation of β-tubulin protein in the transformant suggests that cells maintain equal amounts of α- and β-tubulin by more rapid degradation of the excess subunit not associated with the αβ-dimer or intact microtubules.

The higher ratio of mutant to wild-type β-tubulin results in some exaggeration of the drug-resistant and temperature-sensitive phenotype of the transformant cells compared with the mutant β-tubulin cell line. Although the temperature sensitivity and pattern of resistance to microtubule-depolymerizing drugs are unchanged, the transformant cells are dependent for growth on the presence of these drugs at 37°C, while the mutant is dependent at 40.5°C. At 34°C, where the growth of the transformant is drug independent, the transformant is 10 times more sensitive to the microtubule-stabilizing drug taxol than the Cmd-4 mutant is, and the mutant is more sensitive than the wild type is. Considering that low temperature is known to promote the depolymerization of microtubules (35), these results support the hypothesis that incorporation of the mutant β-tubulin interferes with microtubule function by producing "hyper-stable" microtubules.

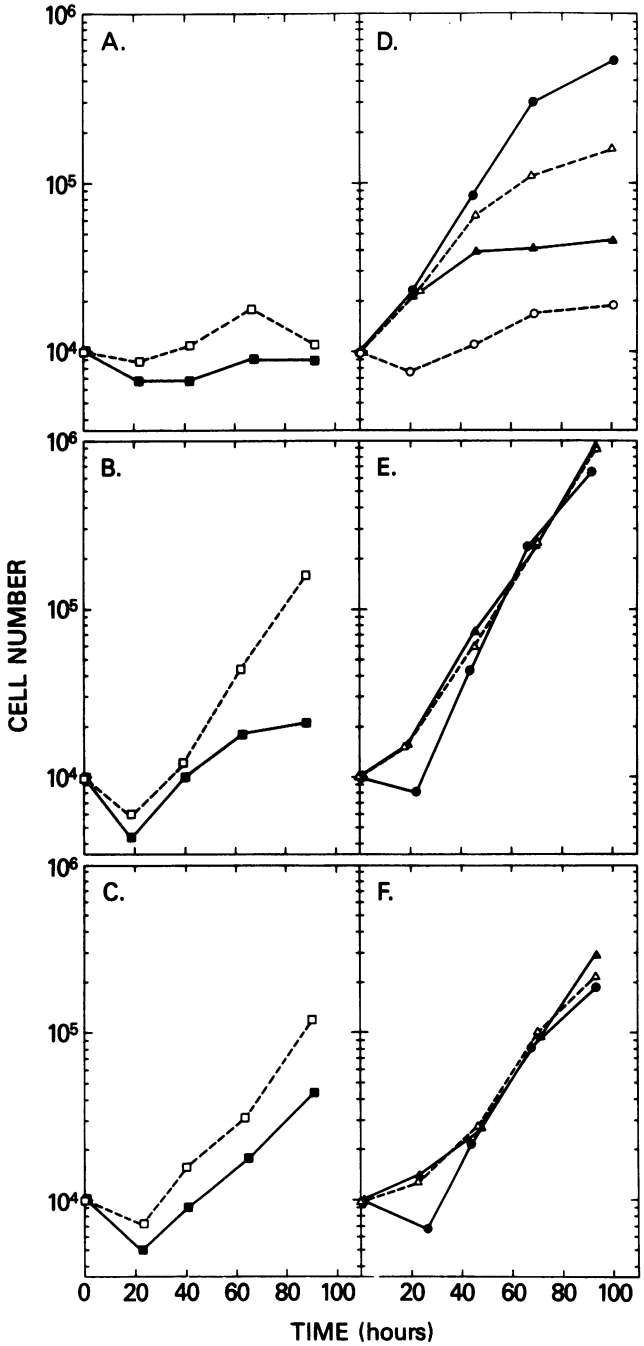

FIG. 5. Growth curves of CHO cells at 40.5°C (A and D), 37°C (B and E), and 34°C (C and F). Initially, 10^4 cells per 30-mm dish (B, E, C, and F) or per 16-mm well of a 24-well dish (A and D) were plated in medium with or without 0.03 μg of Cmd per ml. After various times of incubation, cells were removed with trypsin and counted; total cells per dish were plotted. Transformant cells with (□) or without (■) Cmd; Cmd-4 mutant cells with (△) or without (▲) Cmd; wild-type cells with (○) or without (●) Cmd.

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