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# Isolation of a taxol-resistant Chinese hamster ovary cell mutant that has an alteration in α-tubulin

(microtubules/temperature-sensitive mutant/two-dimensional electrophoresis)

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ABSTRACT **Taxol is a plant alkaloid that has antimitotic activity and appears to stabilize microtubules [Schiff, P. B., Fant, J. & Horwitz, S. B. (1979) *Nature (London)* 277, 665–667]. Taxol-resistant cells were selected from a population of UV-mutagen-treated Chinese hamster ovary cells by a single-step procedure. These mutants have normal morphologies and growth rates but are 2- to 3-fold more resistant to the toxic effects of the drug than the wild-type parent. One out of 20 mutants screened by two-dimensional electrophoresis for chemical alterations in tubulin had an "extra" spot with a more acidic isoelectric point than α-tubulin. This extra spot was shown to be an electrophoretic variant α-tubulin by its copurification with tubulin in crude microtubule-containing preparations and by one-dimensional peptide mapping. The α-tubulin mutant was found to be temperature sensitive for growth, and this property was used as the basis for the selection of revertants. Seventeen temperature-resistant revertants of the α-tubulin mutant were selected for their ability to grow at 40°C and three of these revertants were found to have simultaneously lost their taxol resistance and the electrophoretic variant α-tubulin. These results provide evidence that an alteration in α-tubulin can confer taxol resistance on a mammalian cell line and suggest that α-tubulin is essential for cell viability.**

A number of plant alkaloids such as colchicine, vinblastine, Colcemid, and griseofulvin have been shown to be potent inhibitors of mammalian cell division, presumably through their actions on cellular microtubules (for review, see refs. 1 and 2). These compounds inhibit the polymerization of tubulin *in vitro* and cause the disassembly of microtubules when added to cells in tissue culture. Recently, it has been suggested that taxol, another alkaloid that has antimitotic and antitumor activity, may act by a somewhat different mechanism. In contrast to the other compounds mentioned, taxol acts as a promoter of microtubule assembly *in vitro* and renders microtubules resistant to depolymerization by cold (4°C) and $Ca^{2+}$ *in vitro* and in tissue culture cells (3, 4).

We have described the isolation of Chinese hamster ovary (CHO) cell mutants resistant to colchicine, Colcemid, and griseofulvin. At least one mutant resistant to each of these drugs was found to contain an altered β-tubulin; no alterations were found in the α subunit (5). We now describe the isolation of CHO mutants resistant to taxol. Unlike the mutants described earlier, one of the taxol-resistant mutants carries an altered α-tubulin. This mutant is temperature sensitive for growth. Thus, our genetic analysis supports the biochemical evidence that taxol may act through a distinct mechanism in inhibiting cell division and also confirms the physiological evidence that α-tubulin is essential for cell growth.

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## MATERIALS AND METHODS

**Growth and Labeling of Cells.** CHO ($Pro^-5$) (6) subclone 10001 (parental, wild-type) and mutant strains were grown and labeled with [$^{35}$S]methionine as described (7).

**Mutant Isolation.** Approximately $5 \times 10^6$ parental (10001) cells growing in suspension were plated onto a 100-mm tissue culture dish in 10 ml of minimal essential medium (Flow Laboratories, McLean, VA)/10% fetal bovine serum (Associated Biomedical Systems, Rockville, MD) and incubated at 37°C for 4 hr. The attached cells were washed twice with phosphate buffered saline and then covered with a further 5 ml of phosphate buffered saline. The washed cells were immediately irradiated for 10 sec at a distance of 60 cm with a 15-W General Electric germicidal lamp. Cells were then trypsinized and allowed to grow in suspension culture for 3 days before we proceeded with the mutant isolation. Survival of the cells after mutagenesis was ≈35%.

Taxol-resistant cells were selected by plating $5 \times 10^5$ mutagen-treated cells onto each of five 100-mm dishes containing 20 ml of complete medium and taxol at 0.2 μg/ml (diluted from a stock solution of 0.1 mg/ml in dimethyl sulfoxide). After ≈10 days at 37°C, surviving clones were picked, grown, and recloned in 0.3% agarose. Survivors were found at a frequency of $\approx 10^{-4}$. The Tax-1 mutant that carries an alteration in α-tubulin is our laboratory strain 10576.

**Isolation of Revertants.** The Tax-1 mutant was found to be temperature sensitive for growth. We have made use of this phenotype for the selection of revertants. Briefly, $10^5$ Tax-1 cells not treated with mutagen growing in suspension were plated onto each of ten 100-mm tissue culture dishes and incubated at 40.5°C for 7–10 days. Surviving colonies, found at a frequency of $5–20 \times 10^{-5}$, were picked, recloned, and retested for temperature resistance, taxol sensitivity, and two-dimensional gel pattern.

**Other Procedures.** Drug resistance (5), growth curves (8), two-dimensional gel electrophoresis (9), one-dimensional peptide mapping (10), and purification of assembled microtubules (5) were performed as described.

## RESULTS

**Taxol-Resistant Mutants are 2- to 3-Fold More Resistant to the Drug than Are the Parent Cells.** The sensitivity to taxol of the taxol-resistant mutants and of the wild-type parent was measured by determining the plating efficiency of the cells in various concentrations of the drug. The results show that wild-type parental cells are killed when grown in the presence of taxol

Abbreviation: CHO, Chinese hamster ovary.

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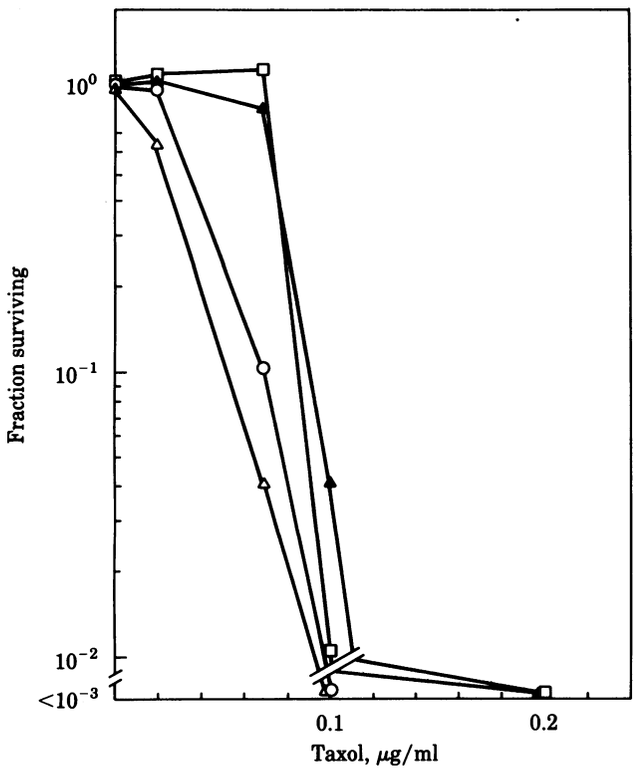


FIG. 1. Cloning efficiency of CHO cells as a function of taxol concentration. Cloning efficiency was determined as described (5). △, Revertant Tax-1 B1; ○, 10001; □, revertant Tax-1 A2; ▲, Tax-1.

at ≈0.1 μg/ml, but the resistant mutants survive in the presence of taxol at >0.1 μg/ml (Fig. 1). This level of increased resistance is similar to that observed in single-step mutants selected for resistance to other drugs that interfere with mitosis (5).

**One Taxol-Resistant Mutant Has an Electrophoretic Variant α-Tubulin.** The 20 taxol-resistant mutants were screened for alterations in tubulin by using two-dimensional gel electrophoresis (Fig. 2). Most of the mutants had two-dimensional gel patterns indistinguishable from that of the wild type, but one mutant, *tax*-1, reproducibly had an "extra" spot of similar molecular weight but more acidic isoelectric point than wild-type α-tubulin. As previously reported (5), the two-dimensional gel pattern of wild-type cells contains single spots representing α- and β-tubulin (Fig. 2*A*). Mutant *tax*-1, however, has a closely spaced doublet in the α-tubulin region (Fig. 2*B*).

Identification of this extra spot as an electrophoretic variant α-tubulin was made by lysing wild-type or mutant *tax*-1 cells in a glycerol-containing buffer that stabilizes intact cellular microtubules (Fig. 2 *C* and *D*). While the wild-type microtubules contain a single α-tubulin, the *tax*-1 microtubules contain two α-tubulin proteins: the normal wild-type protein and an electrophoretic variant α-tubulin that has a more acidic isoelectric point.

Further evidence that the protein identified as an extra spot in the two-dimensional gel pattern of *tax*-1 is α-tubulin is provided by a one-dimensional peptide map (Fig. 3). Wild-type and variant $^{35}$S-labeled α-tubulin were cut from a two-dimensional gel and digested with *Staphylococcus* V8 protease, and the partially digested peptides were resolved on a 15% acrylamide gel. Mutant (lane A) and wild-type (lane B) α-tubulin from the *tax*-1 mutant have identical peptide patterns as demonstrated by their mixture (lane C). These patterns are identical to α-tubulin fragments from the wild-type strain (lane D) and distinct from the patterns obtained for β-tubulin (lane E) or intermediate filament protein (lane F), two other structural proteins that migrate in the same region of the two-dimensional gel.

**The α-Tubulin Mutant Is Temperature Sensitive for Growth.** As most of the β-tubulin mutants we had previously isolated were temperature sensitive for growth (unpublished results), we decided to test the α-tubulin mutant for temperature sensitivity. We found from the growth curves that wild-type and *tax*-1 cells have similar growth rates at 37°C but that the growth of *tax*-1 is much more sensitive to temperature (Fig.

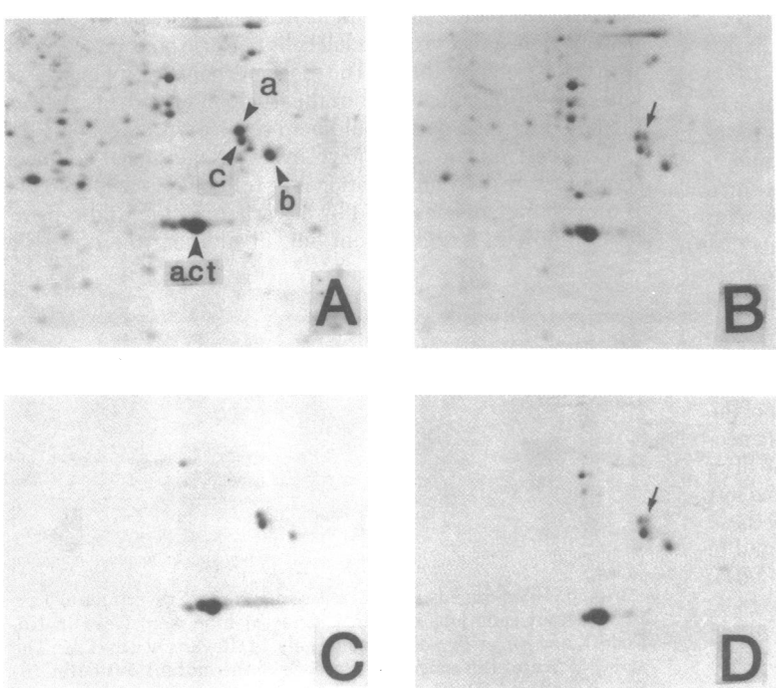


FIG. 2. Two-dimensional gel autoradiograms of wild-type and Tax-1 CHO cells. Cells were labeled in tissue culture with [$^{35}$S]methionine at 20 μCi/ml (1 Ci = $3.7 \times 10^{10}$ becquerels) for 30 min and lysed in $NaDodSO_4$ or used for crude microtubule preparations and run on two-dimensional gels. Only a portion of each gel is shown. Minor differences in the intensities of some spots are due to variations in autoradiographic exposure and photographic reproduction. These and subsequent gels are oriented with the basic side on the left. (*A*) Cell lysate from wild-type parental cells. a, α-Tubulin; b, β-tubulin; c, intermediate filament protein; act, actin. (*B*) Cell lysate from Tax-1 mutant. The arrow indicates the presence of an electrophoretic variant of α-tubulin to the acid side of the wild-type α-tubulin protein. (*C*) Crude microtubule-containing pellet from wild-type cells. (*D*) Crude microtubule-containing pellet from Tax-1 mutant. The arrow indicates the presence of the electrophoretic variant in the microtubule-containing pellet.

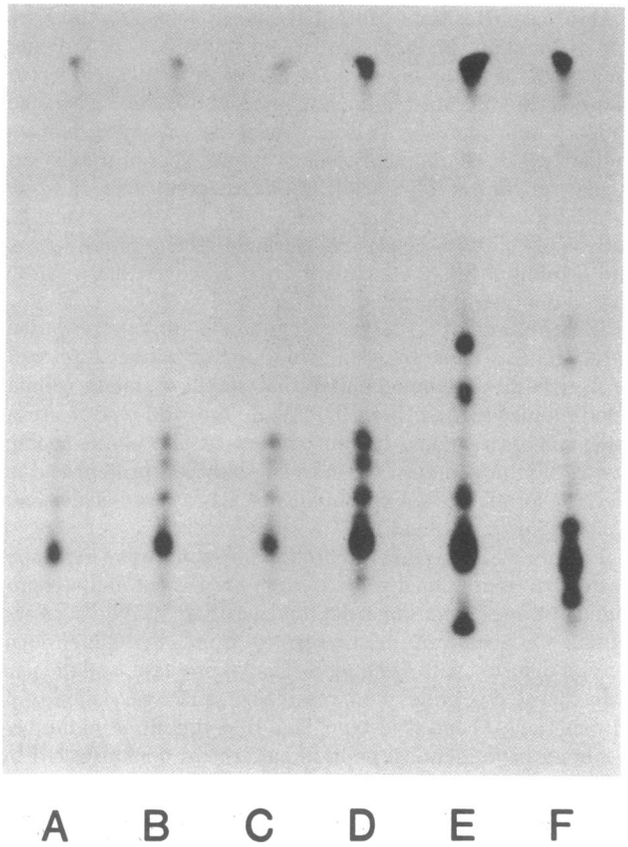


FIG. 3. *Staphylococcus* V8 protease partial digests of wild-type and mutant α-tubulin. Wild-type and Tax-1 mutant cells labeled with [$^{35}$S]methionine were run on two-dimensional gels. Spots corresponding to wild-type and mutant α-tubulin, β-tubulin, and intermediate filament protein were cut out of the gel and run on a 15% acrylamide/NaDodSO$_4$ gel in the presence of *Staphylococcus* V8 protease at 0.1 μg per well. Lanes: A, mutant α-tubulin from Tax-1; B, wild-type α-tubulin from Tax-1; C, a mixture of wild-type and mutant α-tubulin from Tax-1; D, α-tubulin from wild-type cells; E, β-tubulin from wild-type cells; F, intermediate filament protein from wild-type cells.

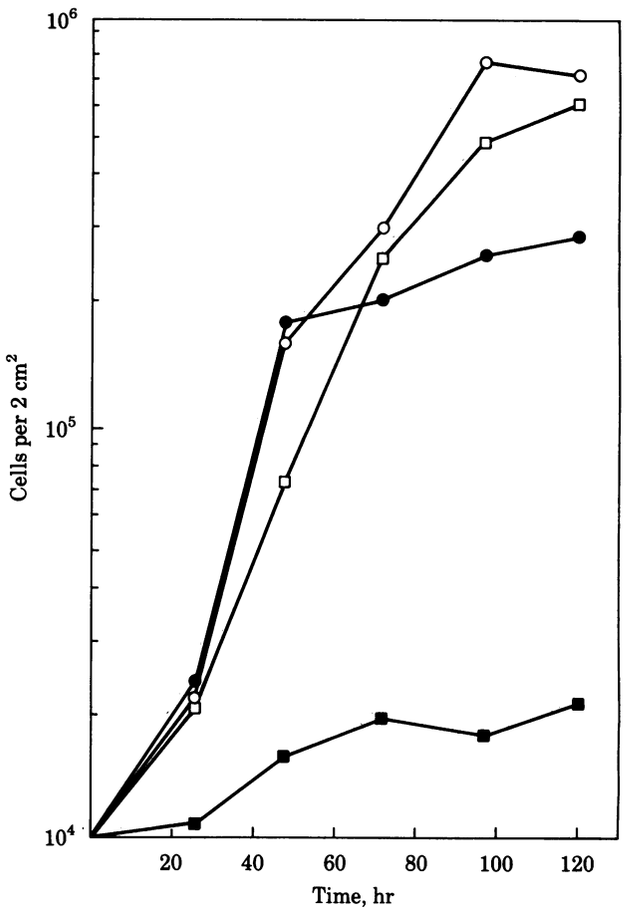


FIG. 4. Growth of mutant and parental CHO cells at 37°C and 40.3°C. Growth curves were obtained as described (8). ○, 10001, 37°C; ●, 10001, 40.3°C; □, Tax-1, 37°C; ■, Tax-1, 40.3°C.

4). The cloning efficiency of *tax*-1 cells was $<10^{-4}$ at 40.5°C, while wild-type cells formed clones with high efficiency at this temperature.

**Revertants Lacking the Altered α-Tubulin Are Taxol Sensitive.** The temperature-sensitive phenotype allowed us to select revertants of *tax*-1 that could grow at elevated temperatures. We reasoned that, if the cells were temperature sensitive because of an alteration in tubulin, then selection of temperature-resistant cells should allow the isolation of revertants that have lost that alteration. Seventeen clones of *tax*-1 able to grow at 40.5°C were isolated and screened for their taxol resistance and their two-dimensional gel patterns. The 17 temperature-resistant revertants fell into two classes on the basis of their two-dimensional gel patterns (Fig. 5). One class of revertants has a two-dimensional gel pattern indistinguishable from that of the *tax*-1 parent (Fig. 5*A*). When tested for their taxol resistance, the cells in this class continued to exhibit resistance to the drug (Fig. 1, Tax-1 A2). Three of the 17 revertants, however, had lost the electrophoretic variant α-tubulin (Fig. 5*B*). When these cells were tested for their taxol resistance, they were found to be as sensitive to the drug as wild-type cells (Fig. 1, Tax-1 B1). This second class of mutants presumably represents intragenic or regulatory revertants that have lost the mutant polypeptide and simultaneously have lost their taxol resistance and temperature sensitivity.

## DISCUSSION

This communication describes the isolation and identification of an α-tubulin mutant in mammalian cells. *tax*-1 was isolated as a taxol-resistant mutant and its existence provides strong genetic evidence that taxol can kill cells because of its interactions with cellular microtubules. The exact mechanism by which taxol kills cells is still unknown, but the isolation of mutants such as the one described here should help resolve this question.

The *tax*-1 mutant was shown by two-dimensional gel electrophoresis to carry an alteration in α-tubulin. In analogy to the β-tubulin mutants described by us earlier (5), the wild-type α-tubulin protein is still present but, in addition, there exists a

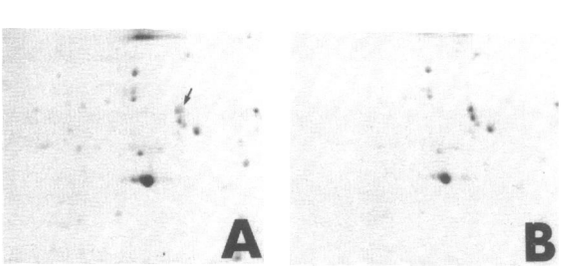


FIG. 5. Two-dimensional gel autoradiograms of revertants of Tax-1. Revertant cells labeled with [$^{35}$S]methionine were lysed in NaDodSO$_4$ and run on two-dimensional gels. (*A*) Revertant Tax-1 A2. The arrow indicates the continued presence of the mutant α-tubulin. (*B*) Revertant Tax-1 B1. The mutant α-tubulin is not expressed.

mutant α-tubulin protein. Also like the β-tubulin mutant, the wild-type protein appears to be present in greater abundance than the mutant protein, as detected in pulse-labeled samples on two-dimensional gels. Although we cannot yet explain this result, one of the possibilities we raised earlier—namely, that there may be more than two loci coding for tubulin in CHO cells, has recently received support from studies using cloned cDNA probes from embryonic chicken brain (11). These studies have provided direct evidence that there are at least four genes each for α- and β-tubulin in chickens. We stress that our own studies cannot be used as proof for the existence of multiple genes. The data, however, are compatible with this interpretation.

Although there is clearly an altered α-tubulin present in *tax*-1, we have not yet shown that the altered α-tubulin results from a mutation in the structural gene for the protein. The possibility that the altered α-tubulin is the result of a posttranslational modification has not been ruled out but seems unlikely as the altered spot is observed in short [$^{35}$S]methionine pulses. The β-tubulin mutants described earlier were clearly shown not to involve posttranslational modifications (5).

The introduction of an altered α-tubulin into the cells apparently results in the formation of microtubules that have different stability than the wild-type microtubules. This presumably explains the temperature sensitivity of *tax*-1 and of the β-tubulin mutants previously described. The precise reason for death of *tax*-1 cells at the nonpermissive temperature is not known at this time. Temperature-sensitive benamyl-resistant mutants of *Aspergillus* have been reported (12) and used to isolate drug-sensitive revertants (13). By using a similar procedure, we have isolated 17 temperature-resistant revertants of *tax*-1. Three of those revertants no longer displayed the altered α-tubulin protein on two-dimensional gels and, when tested for drug resistance, were found to be taxol sensitive. These three revertants may represent intragenic revertants of the original mutation or they may be regulatory revertants that no longer synthesize the mutant polypeptide. They provide strong evidence that the taxol-resistance phenotype in *tax*-1 is expressed through an alteration in the α-tubulin. The remaining 17 temperature-resistant revertants retained their altered α-tubulin and their drug resistance. These revertants may carry further alterations in α-tubulin that do not result in a change in their mobility on two-dimensional gels but do lead to a more stable microtubule structure. Alternatively, these may carry suppressor mutations in other genes that can counteract the effects of the original mutation that result in temperature sensitivity. An example of this type of suppressor could be a mutation in the gene for another protein that interacts with α-tubulin (e.g., β-tubulin, microtubule associated protein, etc.) and compensates for the defect in α-tubulin leading to a more stable microtubular structure. Mutations of this type have previously been described for phage assembly (14) and for microtubule proteins in *Aspergillus* (13).

The isolation of a taxol-resistant mutant that has an alteration in α-tubulin lends further evidence that taxol may inhibit mitosis through a different mechanism than other antimitotic drugs. The β-tubulin mutants described earlier (5) were cross-resistant to colchicine, Colcemid, griseofulvin, and vinblastine. In contrast, the α-tubulin mutant described here appears not to be crossresistant to Colcemid (data not shown).

The *tax*-1 mutant adds a powerful new weapon to our arsenal of mutants that can be used to study the role of microtubules in cellular physiology. It will be of particular interest to determine whether α-tubulin and β-tubulin have any differing roles in the cell and whether a hybrid cell between *tax*-1 and one of the β-tubulin mutants will incorporate both altered subunits into its microtubules.

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