

Isolation of Chinese Hamster Ovary Cell Mutants Requiring the Continuous Presence of Taxol for Cell Division

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ABSTRACT Chinese hamster ovary (CHO) cell mutants resistant to the cytotoxic effects of taxol and requiring the drug for normal growth were isolated in a single step. One of these mutant cell lines, Tax-18, fails to divide in the absence of taxol; instead, the cells become larger, rounder, flatter, and multinucleated. Analysis by flow cytometry indicates that during taxol deprivation there is an accumulation of cells in G₂ + M phase but that the cells are able to leak through the block in the absence of cell division and further increase their DNA content beyond the tetraploid amount. This interpretation is confirmed by karyotype analysis and by time-lapse studies that show cells rounded for mitosis two to five times longer than in wild-type cultures or in Tax-18 cultures grown in taxol. The cells finally attempt to undergo cytokinesis, fail, and spread out again, but as larger cells than before. Tax-18 has a normal growth rate and morphology when grown in taxol even at concentrations three to five times below the selecting concentration of the drug. The cells, however, have increased sensitivity to microtubule-disrupting drugs such as colcemid, griseofulvin, and D₂O. The mutation for taxol auxotrophy behaves recessively in somatic cell hybridization experiments, and the phenotypic reversion rate is $\sim 10^{-5}$ in a nonmutagenized population. Both α - and β -tubulin are present in apparently normal amounts and with normal electrophoretic mobilities on two-dimensional gels. The results suggest that Tax-18 lacks a factor necessary for mitosis and that taxol may be able to substitute for this factor.

Our knowledge of microtubule assembly and function has grown enormously over the past decade. The advances that have been made can be attributed largely to the development of procedures for the in vitro polymerization of microtubules (3, 27, 30) and to the introduction of fluorescent antibody techniques for studying the organization of microtubules in cultured cells (4, 29). These methodologies coupled with both morphological studies (11, 23) and the use of specific microtubule inhibitors (11, 19) have made it possible to elucidate many of the factors that control microtubule assembly and to implicate microtubules in a variety of cellular functions including mitosis, saltatory motion, secretion, morphology, and growth control. In spite of these advances we are still largely ignorant of the mechanism of microtubule involvement in these processes and of the means by which the cell is able to regulate microtubule function.

An approach that should be capable of answering these more subtle questions is one that combines genetics with

biochemistry and morphology. The introduction of a specific lesion into cells followed by a careful characterization of the nature of the lesion and its physiological consequences is a powerful means of studying the role of discrete proteins and cytoskeletal structures in the functioning of a cell. In an attempt to use such an approach, several investigators in the early 1970's selected yeast and *Chlamydomonas* cells resistant to microtubule-active drugs as a way to obtain microtubule mutants (1, 16, 28). The mutants that were isolated, however, did not prove to be very useful because biochemical and morphological defects in the microtubules could not be demonstrated. Later, Davidse and Flach (10) were successful in isolating a large number of benomyl-resistant mutants in *Aspergillus*. Some of these mutants as well as mutants isolated by Morris and his colleagues (21, 26) were subsequently shown to have alterations in α - and β -tubulin. In higher organisms, Ling and his co-workers (18) isolated colchicine-resistant mutants in Chinese hamster ovary (CHO) cells. Although these

early mutants had a permeability defect, the same laboratory was able later to isolate mutants with altered colcemid-binding affinity (14, 17). A similar approach has been used in our own laboratory to isolate CHO mutants with clearly defined alterations in α - and β -tubulin (6-8).

Over the last few years, Horwitz and her co-workers (24, 25) have shown that taxol, an experimental cancer chemotherapeutic drug obtained from the bark of the plant *Taxus brevifolia*, inhibits progression of cells through mitosis by interacting with microtubules. Unlike other microtubule-active drugs that have been described, taxol stabilizes rather than disrupts microtubules, implying that it has a different mechanism of action than the other drugs.

I now report the isolation of CHO cells resistant to the cytotoxic effects of taxol that exhibit the unusual property of requiring the drug for normal growth and morphology. The genetic and biochemical characterizations of these cells are described below. In the accompanying paper (9), the results of a detailed morphological examination of the microtubules in these cells is presented.

MATERIALS AND METHODS

Mutant Isolation: The CHO cell line used in these studies and the conditions for its growth have been previously described (8). Mutant isolation from UV-mutagenized cells was carried out as in an earlier study (6), except that 0.3 $\mu\text{g/ml}$ of taxol was used as the selecting drug concentration. Surviving colonies, which arose at a frequency of 10^{-3} , were picked into taxol-containing medium, grown, recloned, and tested for growth in the presence or absence of the drug.

DNA and Protein Synthesis: The ability of the mutant cells to synthesize DNA and protein was assayed by measuring the incorporation of labeled precursors into the macromolecules. Approximately 2×10^4 mutant cells in 1 ml of complete medium were added to each well of a 24-well dish. Growth rates (measured as described below), protein synthesis, and DNA synthesis were measured at the end of each 24-h period for a total of 4 d on cells incubated in either the presence or absence of 0.2 $\mu\text{g/ml}$ of taxol. Protein synthesis was measured by adding 1 $\mu\text{Ci/ml}$ of [^3H]lysine to the appropriate wells when the cells were first plated and measuring the accumulation of tritium into trichloroacetic acid (TCA)-precipitable material with time of incubation. DNA synthesis was measured by incubating similar cultures with 1 $\mu\text{Ci/ml}$ of [^3H]thymidine in complete medium supplemented with 0.04 mM unlabeled thymidine and again measuring the incorporation of tritium into TCA-precipitable counts. TCA precipitation was carried out by transferring the medium and the trypsinized cells into 5 ml of 10% TCA, incubation at room temperature for 20 min, and then filtering through a glass-fiber filter disk (Whatman GF/B) to catch the precipitate. The filter was then washed twice with 10 ml each of 5% TCA, twice with 10 ml each of 95% ethanol, and then allowed to air dry. Radioactivity trapped on the filter was measured in 5 ml of Econofluor (New England Nuclear, Boston, MA) using a Beckman model LS7500 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Time-lapse Photomicroscopy: Cells for time-lapse observation were plated at ~30% of confluence in T25 flasks (Costar, Data Packaging, Cambridge, MA) containing α -minimal essential medium with or without taxol and allowed to attach overnight at 37°C in a 5% CO_2 atmosphere. The cells were then observed by placing the flasks on the stage of a Leitz Diavert inverted microscope equipped with a thermostatically controlled environment chamber, 32 \times dry objective, RCA 1030H silicon intensifier camera, Panasonic model NV-8030 video tape recorder, and Panasonic model WV-5300 television monitor. The amount of time that cells remained in mitosis was determined by averaging the time from the initiation of cell rounding to the cessation of telophase or "telophaselike" movement for a minimum of five cells.

Flow Cytometry: The DNA content of the cells in a culture was determined using a Phywe model ICP 11 Pulse cytophotometer. Cultures were prepared for analysis by removing the cells from a 70% confluent 100-mm tissue culture dish by trypsinization and combining these cells with the loosely attached mitotic cells removed with the aspirated medium. The combined cells were then collected by low-speed centrifugation (800 g, 5 min), washed twice with cold Dulbecco's phosphate-buffered saline without Ca^{++} or Mg^{++} (PBS), and fixed with 80% ethanol. The cells were then stained with ethidium bromide and mithramycin and analyzed as previously described (2).

Other Procedures: The procedures for measuring growth (12), drug-resistance (8), cell fusion (8), and karyotyping (31) have already been described. An experiment to determine the viability of Tax-18 cells deprived of taxol for varying times was performed by plating an equal number of cells (500) into several replicate 60-mm dishes in complete medium containing 0.2 $\mu\text{g/ml}$ taxol and allowing the cells to attach for 2-3 h at 37°C. The taxol-containing medium was then removed and replaced with taxol-free medium. Taxol was then restored at various times and the dishes were incubated at 37°C for 5-7 d. The number of colonies obtained after taxol deprivation divided by the number of colonies counted on a replicate dish that was not deprived represents the fraction of cells that were able to survive without taxol for a given time.

RESULTS

Selection for Taxol Resistance Yields Cells That Require Taxol for Growth

Our previous attempt to isolate taxol-resistant CHO cells resulted in the discovery of a cell line carrying an alteration in α -tubulin (6). This cell line has normal growth and morphology at 37°C but is unable to grow at higher temperatures that still support growth of wild-type parental cells. Reversion analysis indicated that taxol-resistance and temperature-sensitivity of growth result from the observed alteration in α -tubulin. I have now reisolated 18 taxol-resistant CHO cells from three independent cultures and have found a new class of mutants.

Most of the isolates (11/18) exhibit two- to threefold resistance to taxol but, unlike mutants selected for resistance to colchicine, colcemid, or griseofulvin (8), they show no cross-resistance to other common microtubule-active drugs and are not temperature-sensitive for growth. This class of mutants is still largely uncharacterized. The remaining mutants (7/18) have properties similar to those of the first class but in addition were found to require the presence of taxol for normal growth. Some of the cells in this latter class are only partially dependent on the drug, i.e., they grow better in the presence than in the absence of taxol. At least two isolates, however, demonstrate an absolute requirement for the drug. One of these isolates, Tax-18, was chosen for more detailed analysis.

Mutant Tax-18 Requires Taxol for Cell Division

My visual impression that Tax-18 does not grow in the absence of taxol was quantitated by following the growth kinetics of mutant and wild-type cells incubated in the presence or absence of 0.3 $\mu\text{g/ml}$ of taxol, the selecting drug concentration. As shown in Fig. 1, mutant cells in the presence of 0.3 $\mu\text{g/ml}$ of taxol grow as well as wild-type cells in the absence of the drug. The addition of taxol to wild-type cells or the removal of taxol from mutant cells, however, severely inhibits their growth.

These results were confirmed by observing cells under the microscope using video time-lapse photography (data not shown). Tax-18 cells growing in 0.2 $\mu\text{g/ml}$ of taxol exhibit normal cellular division with a mean mitotic traverse time (from rounding of the cell to cessation of telophase movement) of 50 min. Within 2 h after removal of taxol from the medium, the mean mitotic traverse time increases to 1.5 h and the cells fail to divide. The membrane movements associated with telophase become abnormal and prolonged, but the cell fails to split into daughter cells and flattens out again as a single larger cell. Observation of cells deprived of taxol for longer times reveals similar results except that the time that cells spend in mitosis is further increased to 4.7 h, at 18 h after taxol deprivation.

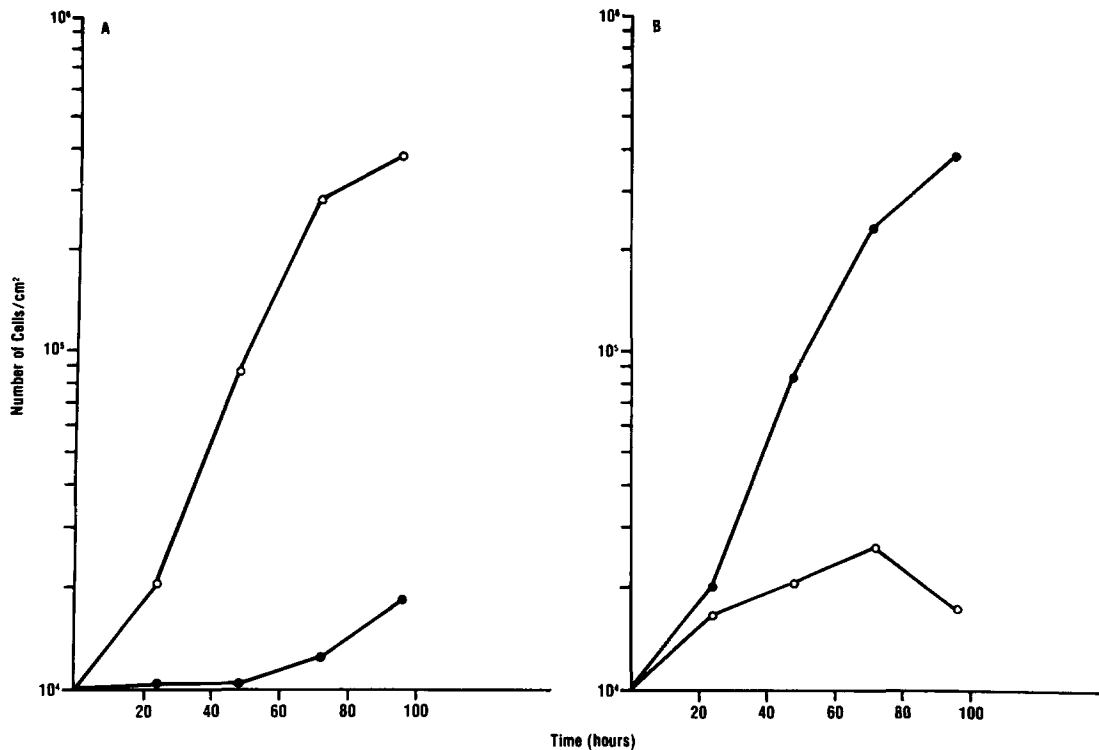


FIGURE 1 Growth curves of wild-type (O) and Tax-18 cells (●) in the absence (A) or presence (B) of 0.3 $\mu\text{g/ml}$ taxol. Cells were trypsinized from replicate wells of a 24-well dish at various times and counted (see Materials and Methods). The vertical axis represents the number of cells in a given well after incubation at 37°C for a given length of time, divided by the surface area of the well.

To test for heterogeneity in the mutant cell population with regard to its dependence on taxol for growth, the plating efficiency of Tax-18 was determined by plating a known number of cells onto dishes, incubating them for 5–7 d at 37°C, and then staining and counting the dishes for viable colonies. The plating efficiency of mutant cells in taxol-containing media was virtually 100% but the plating efficiency of mutant cells in taxol-free media was $\sim 10^{-5}$. This latter number represents the reversion frequency for the Tax-18 cell line (unpublished data). These results show that the Tax-18 cell line is homogeneous in its requirement for taxol.

The minimum amount of taxol that can still maintain growth of Tax-18 cells was determined by plating a constant number of cells in varying concentrations of taxol and then scoring the dishes for growth and morphology (see below) at the end of 3–4 d. The results of this experiment are summarized in Table I. Concentrations between 0.1–0.3 $\mu\text{g/ml}$ taxol were indistinguishable in their ability to promote the growth of the mutant cells. Concentrations of 0.05 and 0.4 $\mu\text{g/ml}$ of taxol were almost as effective but a significant number of cells with aberrant morphology was seen. Concentrations $< 0.05 \mu\text{g/ml}$ or $> 0.5 \mu\text{g/ml}$ were poor in promoting growth of the mutant. Thus, although Tax-18 was selected at 0.3 $\mu\text{g/ml}$ of taxol and although the cells are resistant to 0.4–0.5 $\mu\text{g/ml}$, much lower concentrations (0.05–0.1 $\mu\text{g/ml}$) will maintain growth of the cells.

Tax-18 Deprived of Taxol Assumes an Altered Morphology

One of the most striking changes that can be observed in the mutant during taxol deprivation is its appearance under

TABLE I
Amount of Taxol Required to Maintain Mutant Growth

Taxol $\mu\text{g/ml}$	Growth of Tax-18*
0	—
0.01	—
0.02	+--
0.05	+++—
0.1	++++
0.2	++++
0.3	++++
0.4	+++—
0.5	++—
0.6	+--

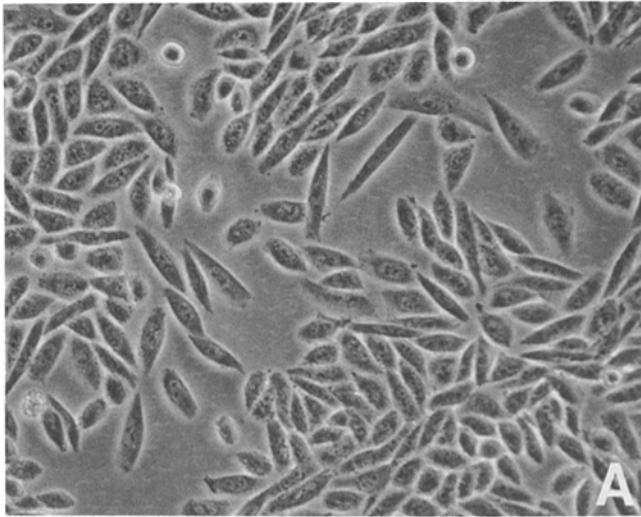
* —, no growth, abnormal morphology; +--, very poor growth, most cells have abnormal morphology; +++—, very good growth, few cells with abnormal morphology; ++++ normal growth and morphology; ++—, good growth, many cells with abnormal morphology.

Approximately 5×10^3 cells were plated in varying concentrations of taxol in 24-well dishes, incubated at 37°C, and scored 3 d later for growth and morphology by microscopic examination.

the light microscope. Fig. 2A is a phase-contrast photograph of Tax-18 cells growing in 0.2 $\mu\text{g/ml}$ taxol. The morphology of the cells under these conditions is very similar to the morphology of the wild-type parental cells. When the mutant cells are deprived of taxol, a profound morphological change occurs (Fig. 2B). The cells become larger, flatter, and rounder and exhibit multiple micronuclei. These changes are apparent at 24 h and become fully manifest by 48 h after taxol deprivation.

The effects of taxol deprivation on the growth and morphology of Tax-18 cells are not reversible. Cell viability was

+ Taxol



-Taxol

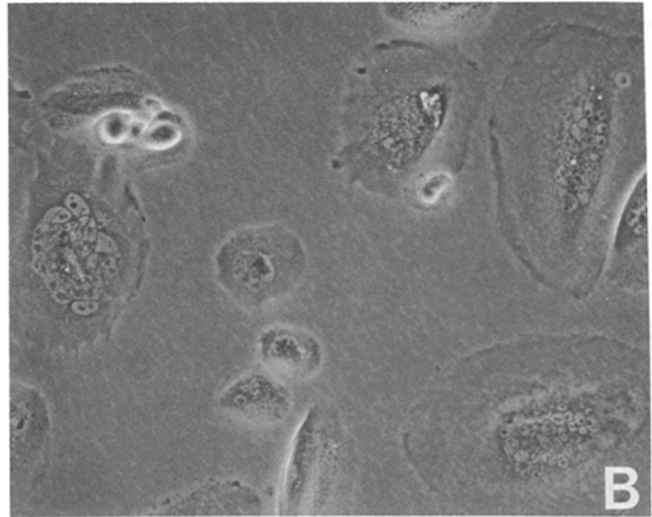


FIGURE 2 Phase-contrast photographs of Tax-18 cells cultured in the continuous presence of 0.2 $\mu\text{g/ml}$ taxol (A), or deprived of taxol for 2 d (B). The cells were photographed with an Olympus IMT microscope with a 20 \times objective.

determined by measuring the plating efficiency of the cells at various times after taxol deprivation. The data plotted in Fig. 3 show that Tax-18 exhibits a rapid loss of ability to form macroscopic colonies when taxol is removed from the medium. This could be due to an effect of taxol on cell metabolism as investigated below, or on the ability of microtubules to function properly during cell division (see also reference 9).

Taxol-deprived Mutant Cells Continue to Synthesize DNA and Protein

Since Tax-18 cells fail to divide in the absence of taxol and since their ability to form macroscopic colonies is rapidly destroyed, it was of interest to determine whether the cells remain alive after long periods of taxol deprivation. Microscopic examination of the cells after prolonged taxol deprivation indicated that the cells continue to synthesize protein since they appear to become larger (see Fig. 2). To test this possibility, the ability of the mutant cells to incorporate [^3H]lysine into protein was measured and the results are shown in Fig. 4. The rate of incorporation of [^3H]lysine (triangles) for the mutant grown without taxol (Fig. 4 B) is somewhat less than what is observed for the mutant grown in taxol (Fig. 4 A), but the cells still incorporate lysine at a very appreciable rate even after 4 d. This protein synthesis is observed even though the cells fail to divide under these conditions (Fig. 4 B, circles). Thus, the cells appear to remain viable even after 4 d without taxol and carry out at least one metabolic process, protein synthesis, at a lowered but very appreciable rate.

Taxol-deprived cells also continue to synthesize DNA as shown by [^3H]thymidine incorporation. Fig. 4 demonstrates that taxol-deprived cells (Fig. 4 B) incorporate thymidine (squares) as efficiently as mutant cells grown in taxol (Fig. 4 A) for the first 48 h, showing that another metabolic function, DNA synthesis, is normal in these cells.

A further indication that taxol-deprived cells continue to synthesize DNA was obtained by using flow cytometry to

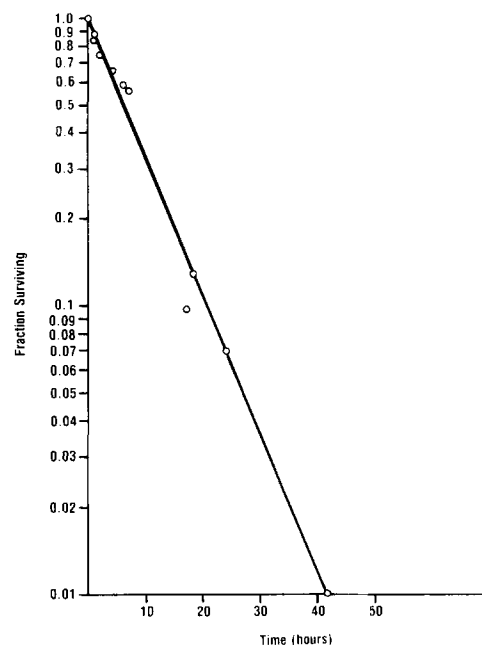


FIGURE 3 Viability of Tax-18 cells deprived of taxol. Approximately 500 mutant cells were plated onto several replicate dishes. The cells were exposed to taxol-free medium for varying amounts of time and then taxol was added back. Colonies were allowed to form and then were counted. The fraction surviving is the number of colonies able to form on a dish deprived of taxol for a given time, divided by the number of colonies able to form on the dish that was kept in taxol-containing medium throughout.

analyze cells at various times after taxol removal. Fig. 5 A shows the histogram of Tax-18 cells growing in taxol. There is a large G₁ phase peak (Channel 30) representing cells with the normal diploid content of DNA, a broad plateau representing cells that are in S phase, and a second, smaller peak (Channel 60) representing $\sim 12\text{--}15\%$ of the total cell population and comprised of cells in G₂ phase and mitosis. This pattern is typical of the histograms obtained with wild-type

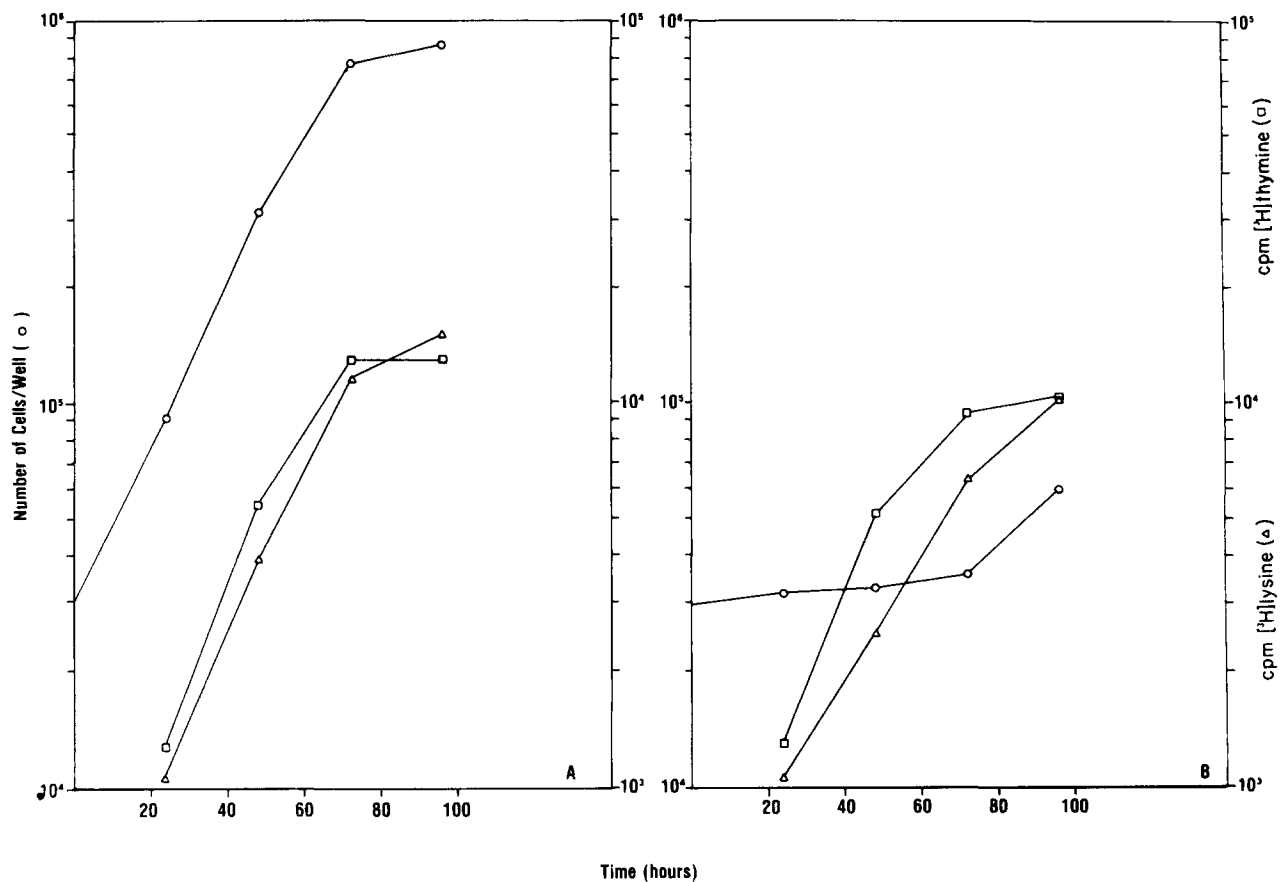


FIGURE 4 Incorporation of [³H]lysine and [³H]thymidine into protein and DNA in Tax-18 cells grown in the presence (A) or absence (B) of taxol. Growth rates (O), [³H]lysine (Δ), and [³H]thymidine (□) incorporation into TCA-precipitable counts were carried out as described in Materials and Methods.

CHO cells. When the Tax-18 cells are deprived of taxol, there is a gradual increase in the G₂ + M phase content to 27% after 7 h (Fig. 5 B), and 50% after 24 h (Fig. 5 C). In addition to the G₂ + M phase peak in Fig. 5 C, there is another peak (arrow, Fig. 5 C) representing an octaploid DNA content. This peak is not found in wild-type cultures or in Tax-18 cultures that are growing in taxol and presumably represents cells that have failed to divide during mitosis but have nonetheless continued to go through the cell cycle and have replicated their tetraploid DNA complement. Tax-18 cells analyzed 48 h after taxol deprivation do not exhibit a further increase in G₂ + M phase or octaploid peaks (Fig. 5 D). This could be due to the possibility that large complements of DNA are unstable and the cell loses extra chromosomes by fragmentation or by an inability to properly replicate all of its DNA. This interpretation is supported by the fact that the histogram in Fig. 5 D shows a much more heterogeneous distribution of DNA in the cells and exhibits a large peak below the G₁ phase peak in the distribution (Channel 12).

A final indication that taxol-deprived Tax-18 cells continue to synthesize DNA and replicate chromosomes is provided by studying the karyotypes of these cells. Representative chromosome spreads of wild-type (a), Tax-18 cells grown in taxol (b), and Tax-18 cells deprived of taxol for 1 (c) or 2 (d) d are shown in Fig. 6. There is a clear increase in the number of chromosomes per cell in Tax-18 when taxol is absent from the growth medium. The proportion of cells with a diploid (21–22), tetraploid (40–44), or octaploid (80–90) chromosomal content is summarized in Table II. These data show

that taxol deprivation skews the karyotype distribution toward higher chromosome numbers but that a significant number of cells with a diploid chromosome content remain even after 2 d without taxol. The reason for the existence of these remaining diploid cells is not well understood. They probably represent a statistical fraction of cells that require an extraordinarily long time to exit mitosis, or they may represent revertant cells that continue to divide during the time course of the experiment. They are not a contaminating subpopulation among the mutant cells since they cannot be removed by recloning Tax-18.

These data argue that taxol-deprived mutant cells are able to continue synthesizing DNA in the absence of cell division. Further evidence that the cells continue to perform cell-cycle specific functions is presented in the accompanying paper (9).

Taxol Dependence Phenotype Behaves Recessively in Somatic Cell Hybridization Experiments

To determine whether Tax-18 cells produce an altered protein that is active in inducing cells to become taxol-dependent, or whether they lack a protein (or other substance) necessary for cell division, the mutant cells were fused with wild-type parental cells and the hybrids were tested for their ability to grow in the presence or absence of taxol. Since it was not known beforehand whether the hybrids would require taxol or whether they would be taxol-resistant, mutant and wild-type cells were first fused and then equal numbers of

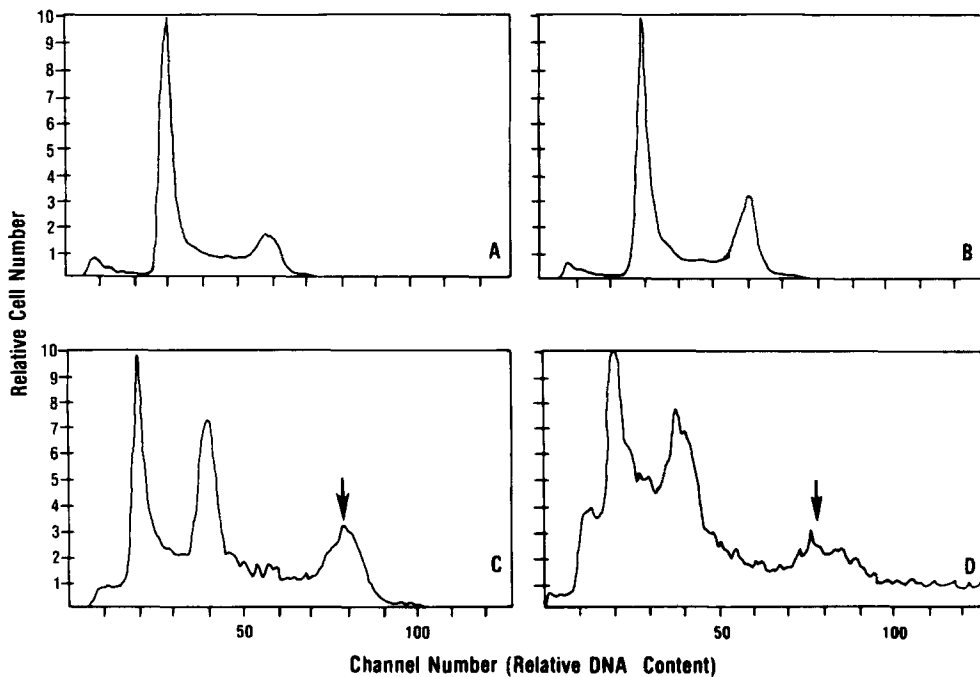


FIGURE 5 Analysis of DNA content in Tax-18 cells by flow cytometry. Replicate cultures of Tax-18 were trypsinized at various times after exposure to taxol-free conditions and then were fixed, stained with ethidium bromide and mithramycin to label their DNA, and run through a flow cytometer. *A*, cells grown in continuous presence of 0.2 $\mu\text{g}/\text{ml}$ taxol; *B*, cells deprived of taxol for 7 h; *C*, cells deprived of taxol for 24 h; and *D*, cells deprived of taxol for 48 h. The gain in *C* and *D* was lowered to allow on-scale visualization of the octaploid DNA peak (arrows). This causes a shift of the peaks to lower channel numbers.

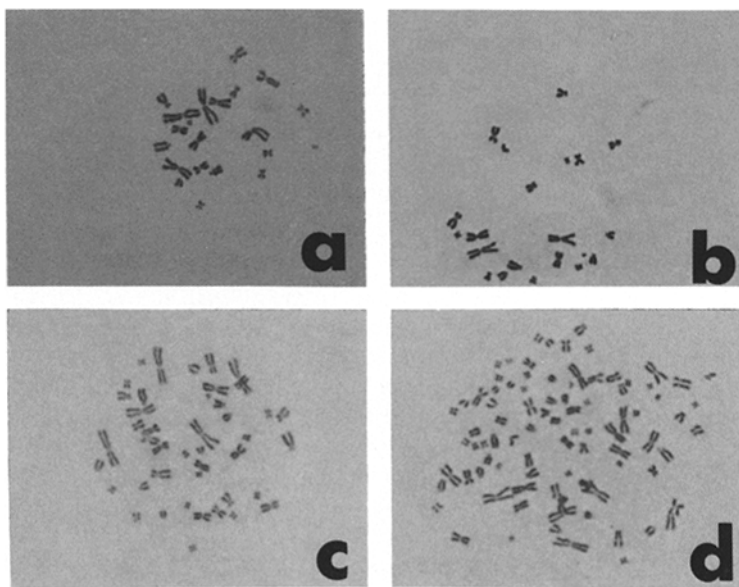


FIGURE 6 Chromosome spreads of Tax-18 cells deprived of taxol for varying amounts of time. *a*, wild-type cells (21 chromosomes); *b*, Tax-18 cells grown in the continuous presence of taxol (21 chromosomes); *c*, Tax-18 cells deprived of taxol for 24 h (42 chromosomes); *d*, Tax-18 cells deprived of taxol for 48 h (84 chromosomes).

fused cells were plated onto replicate dishes containing 0, 0.05, 0.1, or 0.2 $\mu\text{g}/\text{ml}$ of taxol. The results summarized in Table III indicate that mutant/wild-type hybrid cells have lost both their taxol dependency and their taxol resistance. This result suggests that Tax-18 cells lack some factor (protein ?) that can be supplied by the wild-type cells and that is necessary for cell division. That taxol can restore cell division to normal indicates that taxol can substitute for this factor.

That taxol resistance also behaves recessively is more difficult to interpret. Again, if we assume that Tax-18 lacks a factor necessary for spindle assembly and that taxol is able to substitute for that factor, it is reasonable to expect that the cells will tolerate increased amounts of taxol. This is consistent with studies that indicate that taxol inhibits cell division by hyperstabilization of the microtubules (24, 25). If the microtubules are more labile, they should perhaps tolerate more taxol. Evidence for increased lability of the microtubules in

Tax-18 is presented below. In the mutant/wild-type hybrids, the wild-type cells would provide the missing factor so that the hybrids could no longer tolerate increased levels of taxol. Thus, the taxol resistance would behave recessively.

Tax-18 Has Increased Sensitivity to Microtubule-disrupting Drugs

While testing the taxol-resistant cells for cross-resistance to other microtubule active drugs, I noticed that the taxol-requiring mutants appeared to be more sensitive to these drugs than are the wild-type cells. The sensitivity of wild-type and Tax-18 cells to a diverse sampling of microtubule-active drugs is given in Table IV. It may be seen that Tax-18 cells are more sensitive to drugs that normally lead to depolymerization of microtubules (e.g., colcemid, griseofulvin) or drugs reported to stabilize microtubules (e.g., D₂O) (5, 13, 20). The

TABLE II
Chromosome Content of Tax-18 Cells Deprived of Taxol for Varying Amounts of Time

Cells*	Percentage of cells with a given chromosome content		
	Diploid	Tetraploid	Octaploid and higher
WT	96	4	0
Tax-18 +	97	3	0
Tax-18 - 1D	55	45	2
Tax-18 - 2D	18	28	54
Tax-18 - 3D	8	27	65
Tax-18 - 4D	13	17	70

* Cells were karyotyped as described by Worton and Duff (31).
+, cells grown in 0.2 $\mu\text{g}/\text{ml}$ taxol; - 1D, deprived of taxol for 1 d; - 2 D, deprived of taxol for 2 d; etc. 100 metaphase spreads of each cell line were scored for their chromosome content, and the results are expressed as a percentage of the cells examined. Diploid for CHO means 21-22 chromosomes; tetraploid means 38-44 chromosomes; octaploid means 78 chromosomes or greater. WT, wild-type.

mutant and wild-type cells are equally resistant to the non-related drug puromycin, showing that increased sensitivity to toxic drugs is not a general feature of Tax-18 cells. These results suggest that the microtubules in Tax-18 are more labile than they are in the wild-type parental cells even in the presence of the microtubule-stabilizing drug, taxol. The results with D₂O suggest that the requirement for taxol is specific, i.e., other substances reported to stabilize microtubules will not substitute for the drug.

DISCUSSION

Mutants with specific alterations in tubulin or microtubule-associated proteins can provide a potentially rich source of information on the assembly, function, and regulation of microtubules. This approach has been fruitfully exploited by Morris and his colleagues in *Aspergillus nidulans*, where alterations in α - and β -tubulin have been identified and used to show that nuclear migration in that organism is microtubule mediated (22). Recently, Kempfues et al. (15) have described a sterile male mutant in *Drosophila* that lacks a testis-specific β -tubulin, providing genetic evidence for developmentally programmed regulation of tubulin gene expression. In our own laboratory we have described the isolation of CHO cells with alterations in β -tubulin that are resistant to colcemid, colchicine, and griseofulvin (8). A CHO mutant resistant to taxol and carrying an alteration in α -tubulin has also been described (6). These mutants are all temperature sensitive for growth, providing genetic evidence for the necessity of microtubules in cell viability.

In this paper I describe the isolation and preliminary characterization of a novel class of mammalian cell mutants. These mutants, which were selected for resistance to the microtubule-stabilizing drug taxol, were found to require the drug for normal growth. When cultured in the absence of taxol, these cells cease to divide, become larger, flatter, and accumulate many micronuclei. Since the mutant cells require the selecting drug for normal growth and morphology, it is highly unlikely that they represent simple permeability mutants. Taxol must be reaching its intracellular target if the cells are dependent upon the drug for cell division. Data presented in the accompanying paper (9) demonstrate that

the intracellular target of the drug in this mutant is the mitotic spindle.

Both the drug-resistance and the drug-dependence phenotypes in these cells are the result of a single genetic lesion. This conclusion comes from the fact that both phenotypes spontaneously co-revert at a frequency (10^{-5}) consistent with a single mutational event. Interestingly, some revertants have lost their drug requirement but retain the drug-resistance phenotype (work in progress). It is tempting to speculate that this latter class of revertants may contain second site suppressor mutations that affect microtubule stability without negating the ability of the first mutation to confer taxol resistance.

Unlike the β -tubulin mutants described earlier (8), the Tax-18 cells behave recessively when hybridized to the parental wild-type cells, are not temperature-sensitive for growth, and are not cross-resistant to the other microtubule-disrupting agents that I have tested so far. Furthermore, Tax-18 cells

TABLE III
Dominance Analysis of Tax-18/Wild-Type Hybrids*

Concentration of taxol $\mu\text{g}/\text{ml}$	Number of colonies
0	44
0.05	3
0.1	0
0.2	0

* Mutant and wild-type cells were fused, divided into four equal aliquots, and each aliquot was plated onto a 100-mm tissue culture dish containing α -MEM and 0, 0.05, 0.1, or 0.2 $\mu\text{g}/\text{ml}$ taxol. After an overnight incubation, the medium was replaced with medium containing hypoxanthine, aminopterin, and thymidine plus 2 mM ouabain (hybrid selective medium) and the appropriate amount of taxol, and colonies were allowed to grow. The number of hybrid colonies arising on each of the four dishes is reported above.

TABLE IV
Resistance of Tax-18 Cells to Other Microtubule-active Drugs

Drug	Concentration	Growth of wild-type cells	Growth* of Tax-18
Colcemid ($\mu\text{g}/\text{ml}$)	0	+	+
	0.005	+	+-
	0.01	+	-
	0.015	+	-
	0.02	+-	-
	0.03	-	-
Griseofulvin ($\mu\text{g}/\text{ml}$)	0	+	+
	2	+	-
	4	+-	-
	6	-	-
	8	-	-
D ₂ O (%)	0	+++	+++
	20	++	++
	30	++	+
	40	++	+-
	50	+	-+
	60	+-	-
Puromycin ($\mu\text{g}/\text{ml}$)	0	+	+
	2	+	+
	4	+-	+-
	6	-	-
	8	-	-

* Tax-18 cells tested in the presence of 0.2 $\mu\text{g}/\text{ml}$ taxol. Mutant cells deprived of taxol would not grow under any of the above conditions.

+, normal growth; -, no growth and abnormal morphology; +-, slowed growth, many abnormal cells; -+, very poor growth, mostly abnormal cells. For D₂O-treated cells the greater the number of "+", the better the growth.

have a normal two-dimensional gel pattern that exhibits no obvious alterations in the position or amount of α - or β -tubulin (data not shown). Despite my inability to demonstrate structural alterations in tubulin, I believe that the lesion in the taxol-requiring cells affects microtubules. This was first suggested by the fact that these cells are more sensitive to other microtubule-disrupting agents than are the wild-type parental cells and was later shown by experiments indicating that Tax-18 cells are unable to form the mitotic apparatus in the absence of taxol (9). The explanation for the taxol requirement in these cells is still speculative and a discussion of several possibilities is deferred to the accompanying paper.

The physiological consequences of the lesion in Tax-18 are profound. The cells accumulate large numbers of micronuclei. This condition probably arises from an inability of the cells to properly segregate their chromosomes (9). Tax-18 cells do not divide. This was obvious in cultures incubated for several days without taxol observed either by the naked eye or in the phase-contrast microscope. It was further substantiated by plotting growth curves and by following the cells with time-lapse cinematography. The absence of cell division, however, does not prevent the cells from continuing through the cell cycle. Flow cytometric analysis clearly shows that there is a mitotic delay in these cells when they are starved for taxol; but it is also clear that the cells are able to traverse mitosis without cell division and to increase their DNA content to at least an octaploid level. These observations are confirmed by karyotype analysis, which indicates an accumulation of chromosomes during taxol deprivation, and by [³H]lysine and [³H]thymidine incorporation, which shows continued protein and DNA synthesis even in the absence of cell division. Because these metabolic processes continue without cell division, the cells become very large. It is also observed that after several days without taxol the cells begin to die. The total explanation for the death of these cells is not yet available but recent experiments suggest that the cells are unable to process their vastly increased number of chromosomes and literally "choke" on their DNA (experiments in progress).

Experiments are now in progress to find the biochemical lesion in Tax-18 cells by isolating mitotic spindles from wild-type and mutant cells and analyzing the protein composition of the two preparations by two-dimensional gel electrophoresis. A detailed analysis of the Tax-18 mutant should provide important information on the regulation of microtubule assembly and on the role of spindle microtubules in a number of mitotic processes.

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