Retention of Autoregulatory Control of Tubulin Synthesis in Cytoplasts: Demonstration of a Cytoplasmic Mechanism That Regulates the Level of Tubulin Expression

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ABSTRACT Virtually all animal cells rapidly and specifically depress synthesis of new α- and β-tubulin polypeptides in response to microtubule inhibitors that increase the pool of depolymerized subunits, or in response to direct elevation of the cellular tubulin subunit content through microinjection of exogenous tubulin subunits. Collectively, these previous findings have documented the presence of an apparent eucaryotic, autoregulatory control mechanism that specifies the level of expression of tubulin in cultured animal cells. Mechanistically, this regulation of tubulin synthesis is achieved through modulation of tubulin mRNA levels. To dissect further the molecular pathway that underlies this autoregulatory phenomenon, we have now investigated whether enucleated cells still retain the requisite regulatory machinery with which to alter tubulin synthetic levels in response to fluctuations in the pool size of unpolymerized tubulin subunits. Using two-dimensional gel electrophoresis to analyze the patterns of new polypeptide synthesis, we have determined that such cytoplasts can indeed respond to drug-induced microtubule depolymerization by specific repression of new β-tubulin synthesis. Moreover, the response of cytoplasts is, if anything, greater in magnitude than that of whole cells. We conclude that autoregulatory control of β-tubulin gene expression must derive principally, if not exclusively, from a cytoplasmic control mechanism that modulates β-tubulin mRNA stability. For α-tubulin, although the response of cytoplasts after drug-induced microtubule depolymerization is quantitatively less dramatic than that of whole cells, at least part of the regulatory machinery must also be activated through a cytoplasmic regulatory event.

Microtubules, which are composed principally of dimeric subunits of one α- and β-tubulin polypeptide, participate in a diverse spectrum of cellular functions including the establishment of programmed modifications of cell shape during morphogenesis, formation of mitotic and meiotic spindles, and establishment of cilia- and flagella-dependent cell motility. Given the important functions of these dynamic arrays of microtubules whose protein components shuttle between the subunit and polymer forms, it is not surprising that the synthesis of tubulin should be a closely regulated process. As initially reported by Ben Ze'ev et al. (1), marked alterations in the morphology of cultured animal cells after drug-induced microtubule depolymerization are accompanied by repression of new tubulin synthesis. What emerged less expectedly from this work and from our own subsequent efforts (2–6) is the realization that the levels of tubulin synthesis are apparently established in these cells by an autoregulatory pathway closely linked to the pool size of unpolymerized subunits.

Evidence in support of this autoregulatory model has accumulated from a variety of experiments. Treatment of cells with the anti-microtubule drugs colchicine or nocadozole, which induce microtubule depolymerization (see, e.g., references 7 and 8) and a concomitant twofold increase in the pool of depolymerized subunits (9, 10), results in a specific (5–10-fold) repression of new tubulin synthesis (1, 4). On the other hand, treatment of cells with the anti-microtubule drug vinblastine, which induces not only microtubule depolymerization but also precipitation of the depolymerized subunits

(see, e.g., reference 11), yields a mild increase in new tubulin synthesis (1, 4). Moreover, treatment with taxol, a drug that stimulates polymerization and presumably lowers the pool of unpolymerized subunits to a negligible level (12, 13), induces an increase in new tubulin synthesis (4), which under some conditions can be quite dramatic (Pachter, J., and D. W. Cleveland, unpublished observation).

Further support for the autoregulatory model has emerged from elevation of intracellular tubulin content by direct microinjection of purified tubulin subunits to a level comparable to that which would be liberated by endogenous microtubule depolymerization. After such microinjection, tubulin polypeptide synthesis has been found to be rapidly and specifically repressed (5, 14).

Although the molecular mechanism through which this apparent autoregulation is achieved has not yet been identified, we have previously determined that the downregulation of tubulin synthesis in response to an increased pool size of free subunits is accompanied by a rapid loss of tubulin mRNAs (4). Hence, regulation cannot be achieved through a reversible RNA sequestration mechanism. In addition, the apparent rates of tubulin gene transcription in nuclei isolated from cells with normal or elevated pools of tubulin subunits have been found to be indistinguishable (2). This finding suggests either that transcription is not the principal level at which control of tubulin synthesis is exercised or that the isolated nuclei do not faithfully mirror the in vivo situation, presumably as the result of loss of some necessary regulatory factor. Thus, although the sum of the present data points to a relatively novel autoregulatory control mechanism for establishing tubulin synthetic levels, it remains unclear whether regulation is achieved through a nuclear process that involves RNA transcription, RNA processing, and/or RNA transport or, alternatively, through a cytoplasmic event that establishes tubulin mRNA stability.

To dissect further the precise molecular events that underlie tubulin autoregulation, we have now sought to determine whether enucleated cells retain the ability to respond to microtubule depolymerization by specific inhibition of new tubulin synthesis. Under conditions where >95% of cells are enucleated, we report that for β-tubulin the response of these cytoplast preparations is, if anything, greater than that of whole cells. These data indicate that autoregulatory control of β-tubulin gene expression must derive principally, if not exclusively, from a cytoplasmic control mechanism that modulates β-tubulin mRNA stability. Similarly, at least part of the regulatory machinery that modulates α-tubulin synthesis must be localized in the cytoplasm since it is also retained in cytoplasts.

MATERIALS AND METHODS

Cell Culture: Chinese hamster ovary (CHO)[1] cells were grown in Dulbecco's modified Eagle's medium (4,500 mg/liter glucose, Gibco Laboratories Inc., Grand Island, NY) supplemented with 10% fetal calf serum (Gibco Laboratories Inc.), 290 mg/liter glutamine (Gibco Laboratories Inc.), 40 mg/liter proline (Sigma Chemical Co., St. Louis, MO), 110 mg/liter sodium pyruvate (Sigma Chemical Co.) in a 5% CO_2 atmosphere at 37°C. Cells were routinely subcultured 1:10 every 3 d.

Cytoplast Preparation: Cytoplasts were prepared essentially as described by Prescott et al. (15) but with a higher centrifugal force. Specifically, 0.5-in. diameter glass coverslips were rinsed with acetone, dried, washed with 67% nitric acid, 33% sulfuric acid for 2 h, extensively rinsed with tap and distilled water, and stored in a beaker of ethanol. Coverslips were handled individually with fine forceps. In a sterile hood, a coverslip was removed, excess ethanol was removed by aspiration, briefly flamed, and placed into a well of a 4- or 24-well culture plate. Several drops of medium were added to the well to prevent the subsequently added cell suspension from being drawn beneath the coverslip. A dilute cell suspension was then added and cells were allowed to grow for 24 to 48 h. The coverslip was carefully removed and placed cell-side down into a 15-ml Corex tube (Corning Glass Works, Corning, NY) containing ~3 ml of 37°C media supplemented with 50 mM HEPES (Sigma Chemical Co.), pH 7.4, and 10 μg/ml of cytochalasin B (Sigma Chemical Co.). The tube was spun immediately in a prewarmed, fixed angle rotor (Sorvall ss34, E. I. duPont de Nemours & Co., Inc., Sorvall Instruments Div., Newtown, CT) at 6,000 *g* (7,200 rpm) for 35 min. The coverslip was carefully removed and placed into 5 ml fresh medium without HEPES or cytochalasin B and allowed to recover for 30 to 60 min in the incubator before further use. This allows the cytoplasts to respread and assume a characteristic morphology. It is important that, during the enucleation procedure, the temperature remain near 37°C. Lower temperatures produce less efficient enucleation, whereas higher temperatures induce heat shock in the cells. Typically, although 40–60% of the cells are lost from the coverslip, 95% of the remainder are enucleated. In some experiments in which observation of both cells and cytoplasts was desired, the time or speed of centrifugation was slightly lowered to yield a lower efficiency of enucleation and a higher number of remaining cells/cytoplasts. Typically, for analysis of newly synthesized proteins, cytoplasts were incubated in methionine-free media supplemented with [^{35}S]methionine (~1,000 Ci/mmol, New England Nuclear, Boston, MA) to a final concentration of 5 mCi/ml. Labeling was continued for 40 min and cytoplast proteins were then solubilized in SDS-gel sample buffer (16).

In some cases, to ensure absolute purity of the cytoplast preparations, labeled cytoplasts were individually removed from a coverslip using aspiration with a blunt-tipped glass needle attached to a micromanipulator. Approximately 200 cytoplasts were serially collected and then expelled directly into O'Farrell lysis buffer (17) to solubilize total cytoplast proteins.

Autoradiography: Cytoplasts were prepared as described and after recovery from enucleation were rinsed with leucine-free medium. Cytoplasts were labeled in multiwell plates containing 200–300 μl of leucine-free medium supplemented with 50 μCi of 2,3,4,5-[^{3}H]leucine (120 Ci/mmol, ICN Chemical and Radioisotope Division, Irvine, CA) for 40 min. The labeling medium was drawn off, fresh medium was added, and the cells were further incubated for 20 min. The cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 30 min, rinsed in PBS (two times for 5 min), and further fixed and dehydrated in methanol at −20°C. The coverslips were air dried and affixed to microscope slides and allowed to dry. The slides were then dipped in nuclear track emulsion (NTB2 emulsion, Eastman Kodak Co., Rochester, NY). After drying, slides were wrapped individually in aluminum foil and exposed at 4°C for 1 to 4 d. Resultant grains over cytoplasts and cells were photographed on a Leitz inverted phase microscope with a long working distance, 40× phase objective and an Olympus OM2 camera with automatic exposure meter.

Immunofluorescence: For tubulin immunofluorescence, coverslips with cytoplasts were placed in a microtubule-stabilizing buffer (4 M glycerol, 10 mM EGTA, 1 mM MgCl, 25 mM PIPES, pH 6.9) containing 0.1% Triton X-100 for 5 min, the same microtubule stabilizing buffer with 0.5% Triton X-100 for 2 min, and finally into the stabilizing buffer alone for 2 min. The coverslips were then placed into absolute methanol at −20°C for 5 min. The coverslips were rehydrated in PBS for 1 min, and 20–25 μl of a 1:200 dilution of an α-tubulin-specific monoclonal antibody (DM1A, a gift from Stephen Blose, Cold Spring Harbor Laboratories) was added for 1 h at 37°C. The coverslips were washed twice for 5 min in excess PBS at 37°C. About 25 μl of a 1:50 dilution of fluoroscein-labeled goat anti-mouse IgG's (Cappel Laboratories, Cochranville, PA) was then added and allowed to incubate for 40 min. The coverslips were washed (two times for 5 min at 37°C with PBS), dipped in distilled water to remove remaining salts, and air dried. Coverslips were mounted in 90% glycerol, 10% PBS, 5% wt/vol propyl gallate. Immunofluorescence microscopy was performed on a Leitz Ortholux II equipped with a 100-W mercury light source for epifluorescence illuminization, Zeiss 63 × 1.4 numerical aperture oil immersion objective and Leitz H2 cube. Photomicrographs were recorded with a Leitz Vario-Orthomat camera on Kodak Tri-X pan film and developed at 400 ASA.

Two-Dimensional Electrophoresis: Two-dimensional gel electrophoresis was performed essentially as described (17) except that the ampholyte mixture was 1:1:1 of pH 5–5.5, pH 5–7, pH 3–10 (Serva Fine Biochemicals Inc., Garden City Park, NY) for a final ampholyte concentration of 2.4%. Samples initially solubilized in SDS-gel sample buffer were diluted into O'Farrell lysis buffer (17) to which additional urea was then added to bring the final concentration back to 9.5 M. Second-dimension electrophoresis was carried

[1] *Abbreviation used in this paper:* CHO, Chinese hamster ovary.

out using 8.5% polyacrylamide gels as described previously (17). Radioactively labeled proteins were visualized by fluorography (18) using Kodak XAR-2 film.

Quantification of Two-Dimensional Gel Autoradiographs: Autoradiographs of two-dimensional gels were quantified with a Loats Associates, Inc. (Westminster, MD), video densitometry system coupled to an IBM PC computer for analysis of the recorded density patterns. This system uses a Dage video camera linked (DAGE-MTI Inc., Wabash, MI) to an IBM computer to divide each autoradiographic image into a 256 × 256 array of pixels. Each pixel is then assigned by a digitizing board to one of 256 different transmission levels based upon the percent transmission recorded for that pixel.

Calibration of the system was usually achieved by polymerization of successive 1.5-fold dilutions of a [^{35}S]labeled protein sample into distinct, horizontal layers of a polyacrylamide slab gel. The resultant gel was then fluorographed (18), dried, and cut into vertical strips. Each strip thus contained a series of rectangles whose ^{35}S content was known from the original labeled protein sample dilutions. The ^{35}S content of each rectangle was also confirmed by excision of the appropriate regions followed by scintillation counting. A calibration strip was then affixed adjacent to each fluorographed, two-dimensional gel pattern to be analyzed, and the strip plus gel were exposed together on a single x-ray film. Because each calibration rectangle on the strip contained a known number of counts per minute, by digitizing each of the calibration rectangles on the autoradiograph, a direct assignment of counts per minute to a given transmission level could be made for each rectangle. The resultant curve of transmission level versus counts per minute was then fitted by linear regression (coefficient of regression > 0.98). This final curve was then used to calculate a counts per minute value for every transmission level.

Individual polypeptide spots on a digitized autoradiogram were then quantified by defining the perimeter of each spot (using a touch sensitive pad). Each pixel within the perimeter was converted into counts per minute, and the counts per minute were summed to yield a final value for counts within each spot.

Because each autoradiogram to be quantified in this manner contained internal standards for number of counts per minute, corrections for the film response were automatically incorporated.

For direct comparison of quantified autoradiograms of cells or cytoplasts incubated with different anti-microtubule drugs, it was important to correct for any errors introduced by analysis of autoradiographs containing different numbers of total counts per minute and/or exposed to different relative levels. Such corrections were made by quantifying (in addition to quantification of the corresponding tubulin polypeptides) 14 nontubulin polypeptide spots. A scaling factor for comparison of the two different exposures was then calculated by a least squares fitting of the quantified values for each of these 14 polypeptides on the two different images. (In all cases, correlation coefficients exceeded 0.97.) For direct comparison of two gels, all values for the 14 polypeptides and for the tubulin polypeptides were then scaled according to this calculated scaling factor.

RESULTS

Specific Repression of α- and β-Tubulin Synthesis in Whole CHO Cells As the Result of Drug-induced Microtubule Depolymerization

All animal cells investigated to date have been found to respond to colchicine-induced microtubule depolymerization with a repression of new tubulin polypeptide synthesis as measured either by quantitative immunoprecipitation of newly synthesized β-tubulin polypeptides (4) or by quantitation of tubulin mRNA levels (2, 4, 14). As previously shown using two-dimensional gel electrophoresis of newly synthesized proteins in cultured mouse 3T6 cells (1), this depression in synthesis is specific to tubulin polypeptides. Both to demonstrate the overall specificity of this drug-induced response in an additional cell type and to provide a reference point for the pattern of protein synthesis in whole cells, we analyzed the proteins synthesized in CHO cells before and after a 3-h incubation in enough colchicine to induce rapid and complete microtubule depolymerization. After pulse labeling with [^{35}S]methionine, the patterns of polypeptide synthesis (shown in Fig. 1, *A* and *B*) were resolved by two-dimensional gel electrophoresis. As is clear in the figure, not only was the overall level of protein synthesis unaffected by colchicine treatment (as we had reported earlier [4]), but with two striking exceptions the level of synthesis of individual poly-

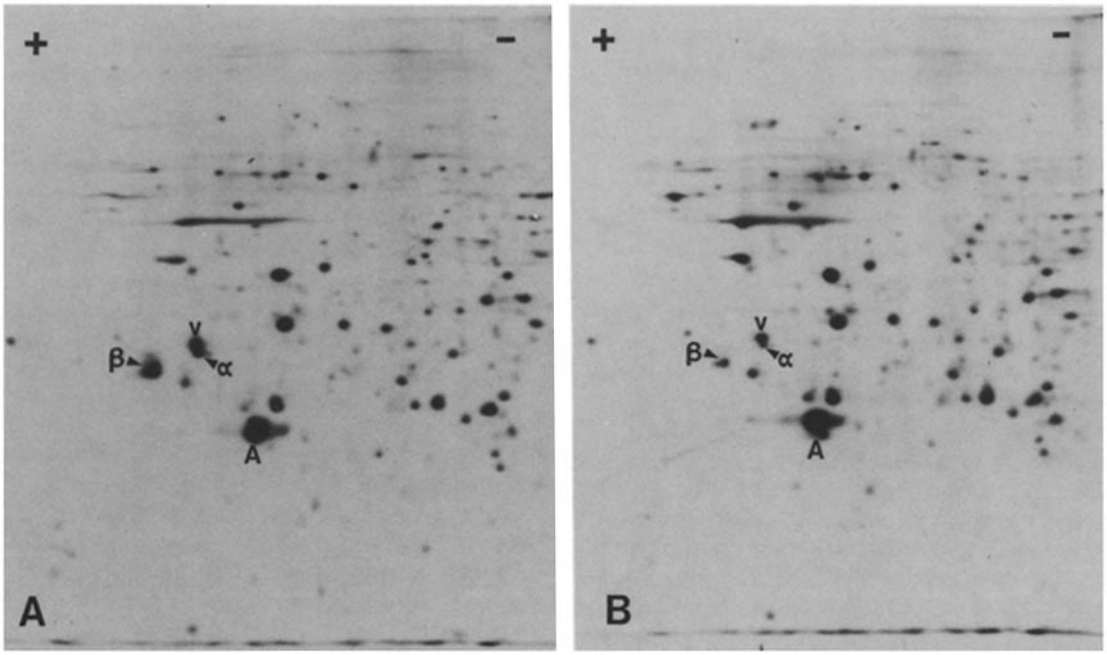

FIGURE 1 Specific repression of new tubulin synthesis in CHO cells in which endogenous microtubules have been depolymerized by treatment with colchicine. CHO cells were incubated in the presence or absence of 10 μM colchicine for 6 h to induce microtubule depolymerization. At the end of drug treatment, both aliquots of cells were labeled with [^{35}S]methionine for 30 min (with colchicine still present for drug-treated cells), and the samples were analyzed by two-dimensional electrophoresis. (*A*) Pattern of protein synthesis in untreated, control cells; (*B*) pattern of protein synthesis in colchicine-treated cells. *A*, actin; *β*, β-tubulin; *α*, α-tubulin; *V*, vimentin. Approximate molecular weights and isoelectric values are as follows: α-tubulin, 55,000, pI 5.2; β-tubulin, 51,000, pI 5.0; vimentin, 58,000, pI 5.1; actin, 43,000, pI 5.3–5.4.

peptides is remarkably constant. Only synthesis of α- and β-tubulin polypeptide is seen to change dramatically (at least at this level of exposure). To confirm this qualitative finding, we quantified the relative levels of synthesis of various polypeptides using computer-assisted, two-dimensional video densitometry as detailed in Materials and Methods. (An example of such a digitized image corresponding to the autoradiograph in Fig. 6*A* is shown in Fig. 6 A′.) The analysis of these results demonstrated that the level of repression of synthesis of new α- and β-tubulin polypeptides is approximately sevenfold for β-tubulin and fivefold for α-tubulin.

Determination of the Molecular Pathway Responsible for Tubulin Autoregulation: Isolation and Characterization of Cytoplasts

To begin to distinguish unambiguously whether the specific repression of tubulin synthesis in response to an elevated subunit pool was achieved through a cytoplasmic regulatory mechanism or through a nuclear event, we exploited the finding of Prescott et al. (15) that, after treatment of whole cells with the microfilament-disrupting drug cytochalasin B, nuclei may be removed from the more adherent cytoplasm by centrifugation. Although immediately after enucleation the morphology of the remaining enucleated cell fragments is highly aberrant (Fig. 2*a*), respreading begins almost immediately after removal of cytochalasin B and appears to be complete within 30–60 min. Except for the lack of nuclei, the recovered cytoplasts look remarkably like cells, having visible organelles and well-defined morphology as shown in the phase-contrast micrograph in Fig. 2*b*.

Moreover, using indirect immunofluorescence with anti-tubulin antibodies, an extensive microtubule network is present in these cytoplasts (see for example Fig. 3*a*). Morphologically, this array of cytoplast microtubules (which has been previously shown to extend at least in part from discrete microtubule initiation sites [19]) appears remarkably similar to the corresponding array in interphase whole cells. In addition, as might be anticipated, this array is sensitive to treatment with colchicine. Fig. 3, *a–d* displays immunofluorescent micrographs of cytoplasts that had been incubated for increasing times in colchicine. At this level of added drug, almost complete depolymerization of the microtubule array is induced within 90 min (Fig. 3*c*). Thus, although a few microtubules in each cytoplast appear stable to colchicine-induced disassembly even after long exposure to the drug (Fig. 3, *c* and *d*), colchicine treatment of cytoplasts results in the depolymerization of an extensive array of microtubule polymers and thereby yields a concomitant, apparent increase in the pool of unpolymerized subunits.

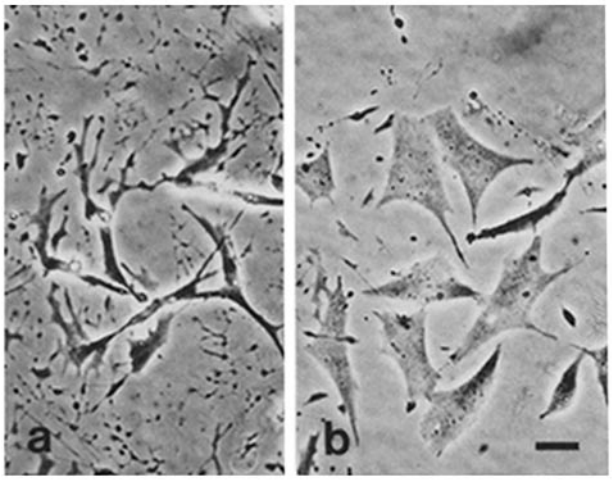

FIGURE 2 Rapid recovery and respreading of cytoplasts after enucleation. Cytoplasts were prepared by treatment with cytochalasin B and centrifugation as described in detail in Materials and Methods. The morphology of the resultant cytoplasts was then followed by phase-contrast microscopy. (a) Appearance of cytoplasts immediately after enucleation. (*b*) Morphology of cytoplasts 60 min after enucleation. Bar, 15 μm. × 360.

Since our initial intent was to determine whether cytoplast preparations could respond to drug-induced microtubule depolymerization with specific repression of new tubulin synthesis, it was important to ensure that cytoplasts could be prepared substantially free from contaminating nucleated cells. To this end, we varied the force and temperature of centrifugation until we identified conditions that consistently resulted in >95% enucleation of CHO cells, where percent enucleation was determined by counting only the larger cytoplasts (i.e., those that were about two-thirds cell size or larger). Fig. 4*a* displays a phase-contrast micrograph of a representative field containing over 100 cytoplasts. No nucleated cell is visible among all of these cell fragments.

The apparent purity of cytoplast preparations is not due to inability to recognize remaining nuclei. For example, when present, cells that retain a nucleus are easily identifiable, as clearly demonstrated in the higher magnification, phase-contrast micrograph in Fig. 4*b*, in which a single nucleated cell (marked with the arrow) is found among a field of cytoplasts. Moreover, the possibility that a substantial population of nucleated cells can escape detection by phase-contrast microscopy was eliminated by staining cytoplast preparations with the DNA-binding dye ethidium bromide. As demonstrated in Fig. 4*c*, nuclei are readily observable with fluorescence optics after ethidium bromide staining. In all instances, the calculated fraction of nuclei-containing cells determined either by phase morphology or by fluorescence was indistinguishable.

Finally, although it was clear that our cytoplast preparations were substantially free of nucleated cells, the possibility remained that the viability of the cytoplasts was impaired enough that a significant fraction of the newly synthesized polypeptides obtained in metabolic labeling experiments might actually derive from the contaminating nucleated cells. To test whether this possibility might be correct, we briefly incubated cytoplast preparations in [^{3}H]leucine in order to label newly synthesized proteins. To detect whether the bulk of the radiolabel was incorporated into the cytoplasts or into whole cells, we then dipped the preparations in a photographic emulsion and subjected them to autoradiography. The results of three independent experiments are shown in Fig. 5. In each part of the figure a solid arrow marks the location of a single nucleated cell in a cytoplast preparation. In each instance, the density of autoradiographic grains over cytoplasts and over the whole cells is qualitatively indistinguishable. The presence of grains over cytoplasts is due to true incorporation of [^{3}H]-leucine into new polypeptides and not to nonspecific trapping of radiolabel since grains are localized only over cells and cytoplasts, and an occasional (presumably nonviable) cytoplast shows no visible grains at all (open arrow in Fig. 5). Overall, since cytoplasts comprise >95% of the total material in our cytoplast preparations and each cytoplast synthesizes

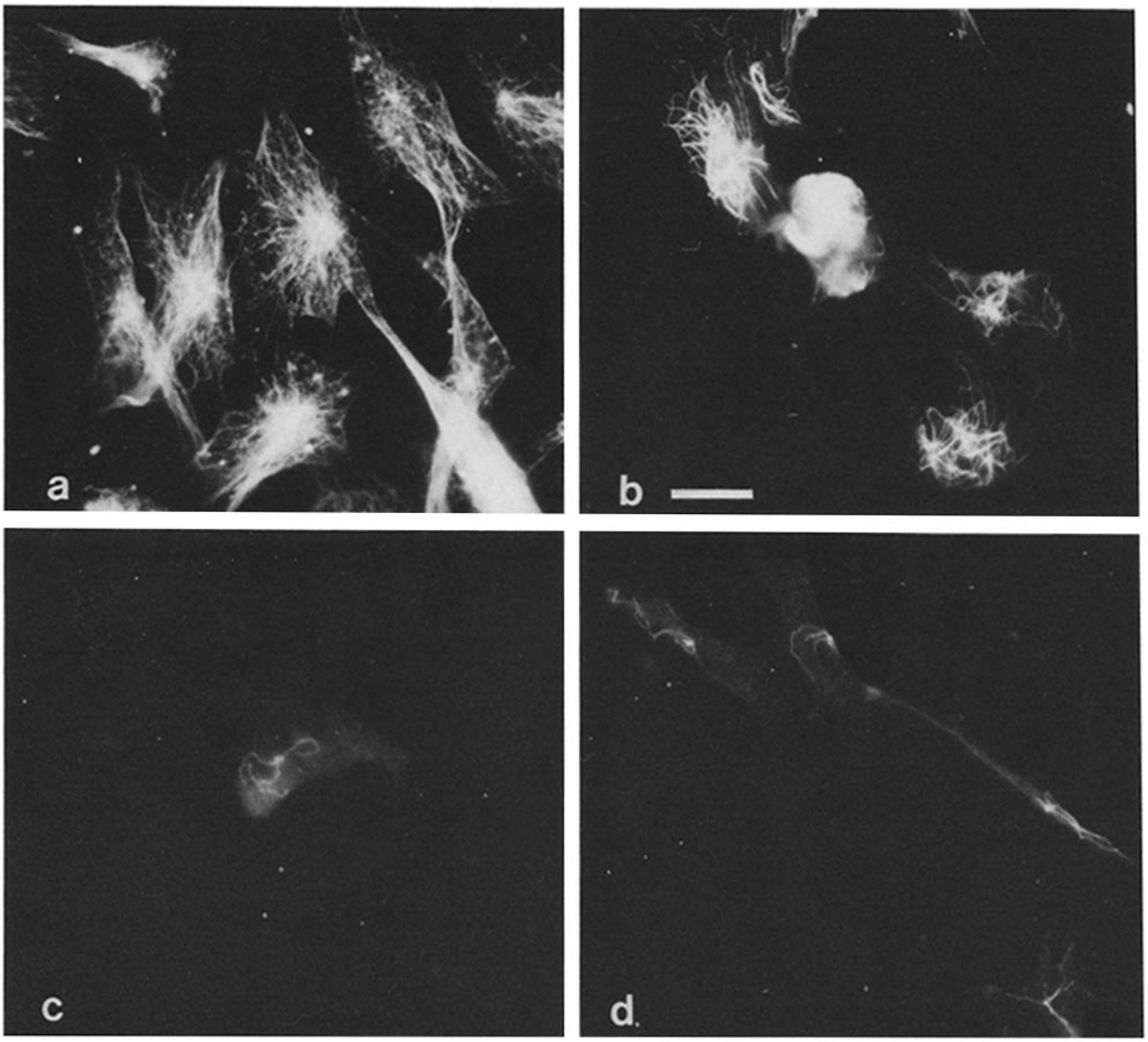

FIGURE 3 Microtubule arrays in cytoplasts before and after treatment with colchicine as determined by indirect immunofluorescence. After a 60-min recovery period after enucleation, cytoplasts were treated with 10 μM colchicine for varying times. Samples were fixed with formaldehyde after extraction with a detergent-containing, microtubule-stabilizing buffer and then incubated with a monoclonal antibody to α-tubulin. Finally, a fluorescein-labeled goat anti-mouse secondary antibody was used to visualize bound tubulin antibody. Shown are the immunofluorescent patterns of microtubules observable after incubation in colchicine for (*a*) 0 min, (*b*) 30 min, (*c*) 90 min, and (*d*) 180 min. Bar, 15 μm. × 800.

polypeptides at a rate comparable to that of whole cells, the vast majority of newly made proteins in such preparations must derive from cytoplasts rather than from whole cells.

Cytoplasts Respond to Drug-induced Microtubule Depolymerization by Specific Repression of New Tubulin Synthesis

To determine whether cytoplast preparations retained the ability to respond to colchicine treatment with specific repression of tubulin synthesis, newly synthesized proteins in cytoplasts were briefly labeled with [^{35}S]methionine and then examined by two-dimensional gel electrophoresis. The synthetic patterns of control cytoplasts to which colchicine was not added are shown in Fig. 6, *A*, *B*, and *C*, for cytoplasts labeled at 0, 90, or 180 min respectively, following a 60-min respreading period post-enucleation. Keeping in mind that the cytoplasts must lose the ability to synthesize new polypeptides since RNAs lost through degradation cannot be replaced, the patterns of protein synthesis during this 3-h period after enucleation are remarkably constant. However, with increasing time after enucleation the relative levels of synthesis of several proteins in Fig. 6 decline in amount (compare the patterns from non-drug-treated cytoplasts in Fig. 6, *A–C*). This is not really surprising since such polypeptides can be inferred to be encoded by RNAs with inherently short (<2 h) half lives (at least in cytoplasts).

Nonetheless, despite such differences between cells and cytoplasts, by comparison of the synthetic patterns in control and colchicine-treated cytoplasts, it was still possible to determine whether cytoplasts retained the requisite molecular machinery for modulation of tubulin synthesis after drug-in-

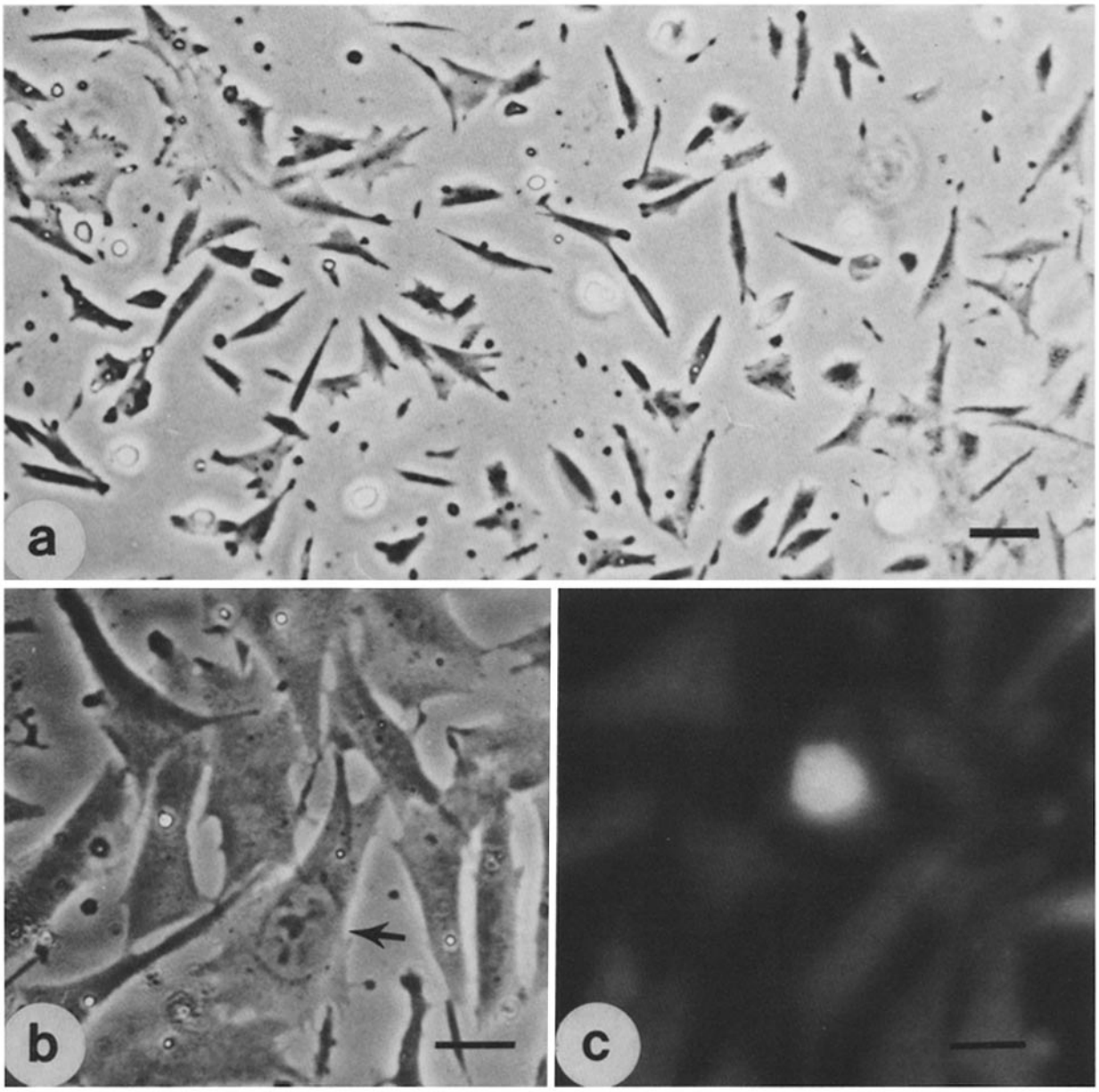

FIGURE 4 Demonstration that cytoplast preparations are virtually free of whole cells. *a* shows a phase-contrast micrograph of a large field of cytoplasts obtained by the standard enucleation protocol. Among the >100 cytoplasts there are no cells present with visible nuclei. *b* displays a higher magnification phase-contrast micrograph of a field of cytoplasts in which a single nucleated cell (arrow) is easily distinguished. *c* shows a fluorescence micrograph of a cytoplast preparation that was treated with ethidium bromide to stain nuclei present in residual cells. Again, a single nucleated cell is clearly detected. Bars, (*a*) 50 μm; (*b* and *c*) 15 μm. (*a*) × 200; (*b* and *c*) × 730.

duced microtubule disassembly. Through the use of [^{35}S]-methionine to pulse label newly synthesized polypeptides, the pattern of protein synthesis in control cytoplasts and in colchicine-treated cytoplasts was investigated. Fig. 6*B* displays a two-dimensional gel analysis of newly synthesized proteins from control cytoplasts 90 min after respreading. Fig. 6*D* displays the analogous pattern for cytoplasts to which colchicine was added for 90 min after respreading. Qualitatively and quantitatively, the patterns are very similar with the obvious exception that new β-tubulin synthesis is markedly reduced in the colchicine-treated sample. Moreover, Fig. 6, *C* and *E* show the corresponding patterns of newly synthesized polypeptides in control and drug-treated cytoplasts after 3 h of drug-induced microtubule depolymerization; selective repression in synthesis of new β-tubulin polypeptides is even more clearly visible in this example.

To quantify these findings, two-dimensional densitometry was used to analyze each of the autoradiographs shown in Fig. 6, *A–E*. An example of the digitized image corresponding to Fig. 6*A* is shown in Fig. 6*A′*. The resultant digitized values were scaled by linear regression to correct for the somewhat greater exposure of the colchicine-treated samples (Fig. 6, *D* and *E*) than of the control cytoplasts (Fig. 6, *B* and *C*). These calculations indicated that the relative exposures of Fig. 6, *A–E* were 1:0.79:0:83:1.30:1.92, respectively. The areas corresponding to α-tubulin, β-tubulin, vimentin, and 13 other polypeptides were identified and quantified, and the results recorded in Table I. As is evident in the table, β-tubulin

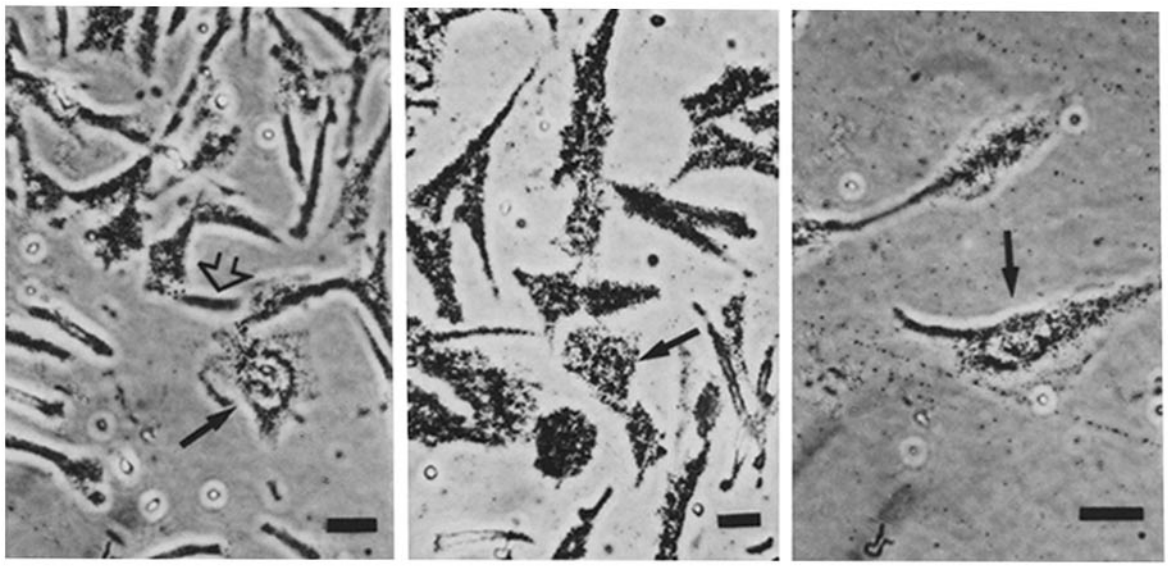

FIGURE 5 Autoradiography of [^{3}H]leucine-labeled preparations reveals the level of protein synthesis to be comparable in cytoplasts and in whole cells. Coverslips from three independent enucleation experiments were labeled with [^{3}H]leucine, dipped in a photographic emulsion, and the autoradiographs were developed. Each cytoplast sample shown includes a nucleated cell (indicated by the solid arrows). The open arrow in the left panel identifies a cytoplast that has incorporated no leucine and is apparently nonviable. Bar, 15 μm. (*Left to right*) × 450; × 380; × 570.

synthesis was depressed by ~12-fold in the drug-treated sample. When we compared this with quantitative analyses of corresponding two-dimensional gels of proteins synthesized in whole cells (one example of which is displayed in Fig. 1) or with quantitative immunoprecipitations from whole cells (4), we discovered that the magnitude of the decline in β-tubulin synthesis upon colchicine-induced microtubule depolymerization is ~1.7-fold greater in cytoplasts than in whole cells.

Similarly, when the autoradiographs from control and colchicine-treated cytoplasts (compare Fig. 6, *C* and *E*, respectively) were corrected for the 2.3-fold greater exposure of the colchicine-treated sample (see above), synthesis of α-tubulin was also found to be depressed in the colchicine-treated cytoplasts. However, in this instance the magnitude of the repression was quantitatively only two- to threefold, a level that is only ~50% that in whole cells.

On the whole, colchicine-induced depression of new synthesis appeared reasonably specific to the tubulins since of the 13 additional polypeptide spots analyzed, synthesis of the tubulins was most markedly affected. Of the remaining polypeptides quantified, only spot 5 deviated substantially (>45%) in value. We conclude that, although differences in nontubulin polypeptides are observed, the principal effect of colchicine-induced microtubule depolymerization in cytoplasts is repression of new tubulin synthesis.

To determine whether this loss of new tubulin synthesis in cytoplasts could also be induced by treatment with additional anti-microtubule drugs, we analyzed the patterns of protein synthesis in cytoplasts incubated with the drugs nocodazole and taxol. Nocodazole, like colchicine, induces microtubule depolymerization and increases the pool of depolymerized subunits. Autoradiographs of the resultant two-dimensional gels are shown in Fig. 7, *A* and *C* for control and nocodazole-treated cytoplasts, respectively. Again, β-tubulin synthesis is specifically depressed in the nocodazole-treated cytoplasts. Quantitatively, this repression was found to be threefold using two-dimensional densitometry. This specific modulation of the β-tubulin synthetic level is not simply due to disruption of microtubule function by all anti-microtubule drugs. Rather, consistent with the original autoregulatory hypothesis, treatment of cytoplasts with the drug taxol, which induces additional microtubule polymerization and hence lowers the pool of depolymerized tubulin subunits, yields the pattern of new protein synthesis displayed in Fig. 7*B*. In this instance, the level of new β-tubulin synthesis is mildly elevated with respect to other cellular proteins. This qualitative observation is also supported by densitometry, which reveals a 30% relative increase in new β-tubulin synthesis in taxol-treated cytoplasts. However, even on longer exposures in which α-tubulin polypeptides were easily visible, no significant change in α-tubulin levels was apparent in this experiment.

Apparent Regulation of Tubulin Synthesis in Cytoplasts Cannot Be Due to Contaminating Nucleated Cells

Although we have shown our cytoplast preparations to be substantially free of nucleated cells (Fig. 4) and further that the vast majority of labeled proteins are synthesized by cytoplasts rather than by residual nucleated cells (Fig. 5), to remove any possible doubt as to the ability of cytoplasts to regulate tubulin synthesis in response to a drug-induced increase in the pool of depolymerized subunits, we have also used a protocol to collect individual cytoplasts. To do this, we have used an apparatus designed for microinjection of animal cells, but instead of fine-tipped glass needles to inject cells, we have used blunt-tipped needles to collect individual cytoplasts by aspiration. In the actual experiment, after labeling was done with [^{35}S]methionine, ~200 cytoplasts were serially collected. At this point they were expelled into O'Farrell lysis buffer (17), and the newly synthesized proteins were analyzed by two-dimensional gel electrophoresis. Fig. 8*A* displays the resultant pattern for control cytoplasts, whereas

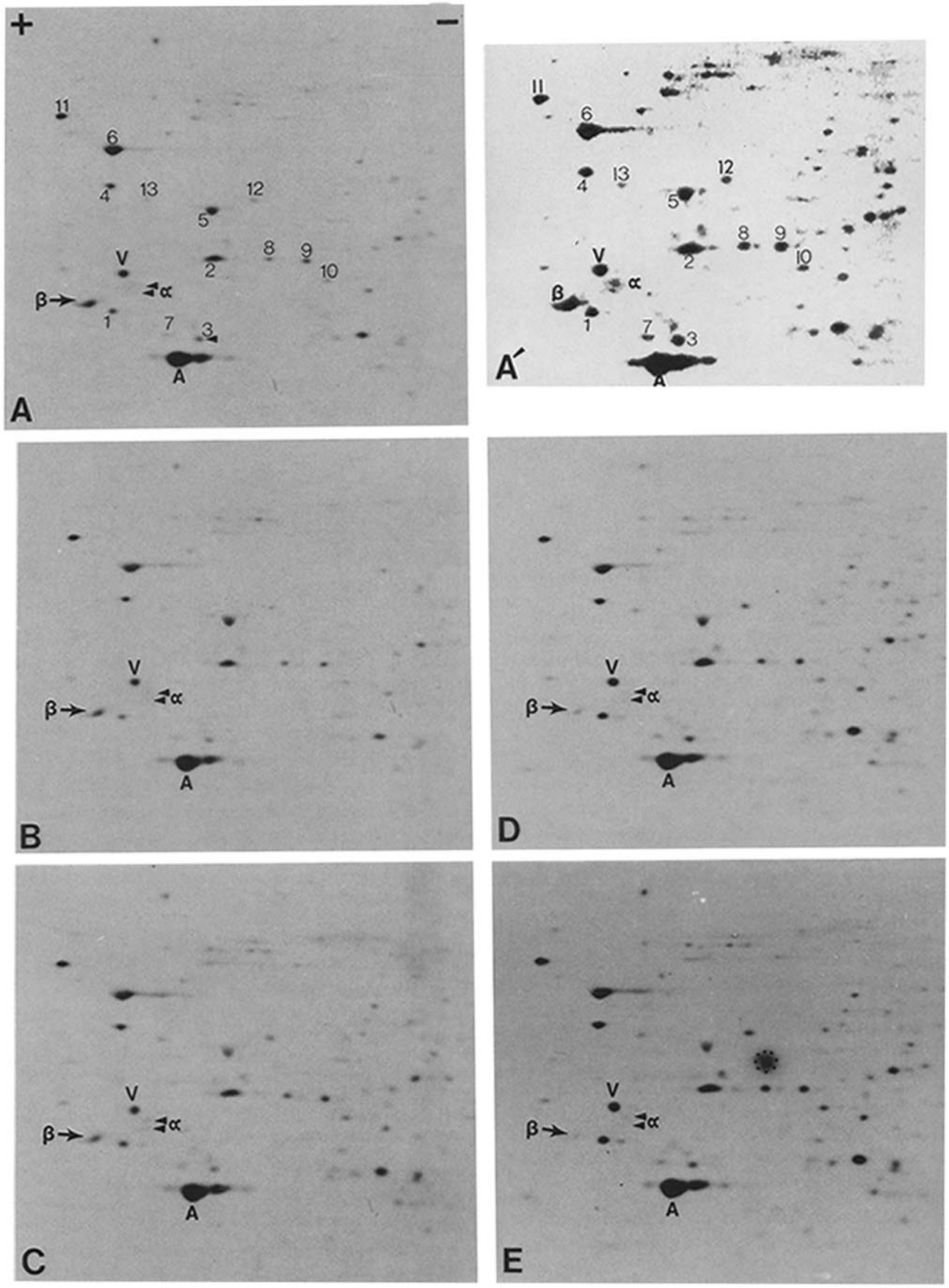
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TABLE I. *Quantification of Various Polypeptide Synthetic Levels in Cytoplasts**

Polypeptide	Colchicine			+ Colchicine		% Difference between colchicine-treated and control**	
	0 time	90 min‡	180 min§	90 min‖	180 min¶	90 min	180 min
β-Tubulin	13.6	13.0	12.6	2.3	1.0	−465	−1,160
α-Tubulin	2.9	4.9	4.9	2.9	1.3	−68	−280
Vimentin	11.0	11.3	10.8	12.2	10.1	+7	−7
1	4.7	4.7	4.7	7.6	6.4	+38	+27
2	15.2	17.0	16.4	21.6	16.2	+21	−1
3	4.0	4.2	4.1	4.5	3.8	+7	−8
4	6.5	7.4	7.7	7.9	7.5	+6	−3
5	13.1	14.9	14.9	10.1	5.2	−44	−190
6	35.2	35.2	35.3	24.4	24.8	−44	−42
7	1.4	2.0	1.8	2.4	1.6	+17	−12
8	3.4	4.7	4.4	4.6	5.3	+2	+17
9	4.4	4.6	4.4	4.6	4.6	+0	+4
10	1.7	1.9	2.0	2.3	3.1	+17	+35
11	10.3	11.9	ND	9.0	8.8	−32	ND
12	2.0	2.4	2.3	2.9	3.2	+17	+28
13	1.0	1.1	1.2	1.1	1.3	+0	+8

* Quantification in arbitrary units of the autoradiographs shown in Fig. 6.
‡ Data have been scaled by a factor of 1.27 to correct for level of exposure.
§ Data have been scaled by a factor of 1.2 to correct for level of exposure.
‖ Data have been scaled by a factor of 0.77 to correct for level of exposure.
¶ Data have been scaled by a factor of 0.52 to correct for level of exposure.
** Calculated as ([final value − initial value]/final value) × 100.

Fig. 8*B* shows the corresponding pattern from cytoplasts treated for 6 h with colchicine. Clearly, as we have already seen in Fig. 6 for bulk cytoplasts, β-tubulin synthesis is specifically repressed as a consequence of drug-induced microtubule depolymerization. Analysis of the patterns by densitometry reveals that this repression is approximately sevenfold. Unfortunately, in this experiment α-tubulin polypeptides were not sufficiently resolved from vimentin to permit quantitation of α-tubulin synthetic levels.

Apparent Regulation of Tubulin Synthesis in Cytoplasts Is Due to a True Change in the Level of Tubulin Synthesis and Not to Instability of Tubulin Polypeptides in Cytoplasts

Because we used relatively long labeling times (40 min) to analyze the patterns of newly synthesized proteins in cytoplasts, the observed decline in the amount of tubulin polypeptides synthesized in colchicine- or nocadozole-treated cytoplasts either could be the result of retention of the appropriate tubulin regulatory machinery in cytoplasts (particularly for β-tubulin) or alternatively could be due to a markedly increased rate of tubulin polypeptide degradation induced in cytoplasts by treatment with these anti-microtubule drugs. To eliminate the latter possibility, we sought to determine the half-life of tubulin polypeptides in cytoplasts in both the presence and the absence of anti-microtubule drugs. To do this, we labeled cells for 18 h with [^{35}S]methionine before preparation as cytoplasts. At various times after enucleation, two-dimensional gels were used to examine the remaining prelabeled polypeptides. Autoradiographs of the resultant patterns are shown in Fig. 9 for equivalent numbers of cytoplasts taken immediately after enucleation (Fig. 9*A*), control cytoplasts 6 h after enucleation (Fig. 9*B*), or cytoplasts treated for 6 h with colchicine (Fig. 9*C*). Clearly, neither α- nor β-tubulin polypeptides are lost relative to other polypeptides over this 6-h period. This qualitative finding was confirmed by quantitative densitometry, which revealed no significant loss of tubulin polypeptides over the 6-h period examined. Thus, although an accurate half-life of tubulin polypeptides in cytoplasts cannot be determined from these data, it is clear that the half-life is quite long as compared with the time scale of the cytoplast experiments presented in Figs. 6–8; hence, differential polypeptide half-life cannot explain the retention of tubulin regulation in cytoplasts.

DISCUSSION

Modulation of the level of new tubulin synthesis in response to alterations in the pool of unpolymerized tubulin subunits is a regulatory event that has been documented in all animal cells investigated. Regulation is achieved rapidly through changes in cellular tubulin mRNA levels. A variety of previous

FIGURE 6 Resolution of newly synthesized proteins in cytoplasts treated with colchicine reveals down regulation of β-tubulin synthesis. After recovery for 60 min, cytoplasts were incubated in the presence or absence of 10 μM colchicine for increasing periods, and newly synthesized proteins were labeled with [^{35}S]methionine for the final 40 min. The labeled proteins were then analyzed by two-dimensional gel electrophoresis. *A*, *B*, and *C*, respectively, represent synthesis patterns in untreated, control cytoplasts after 0, 90, or 180 min. *D* and *E* represent synthesis patterns in cytoplasts treated with colchicine for 90 or 180 min, respectively. The circled spot in *E* represents an artifactual spot of ^{32}P contamination, inadvertently introduced during gel drying. *A*, actin; *β*, β-tubulin; *α*, α-tubulin; *V*, vimentin. Spots labeled 1–13 represent reference proteins whose intensities were densitometered. A′ is a black and white photograph of a color image representing the video display of the digitized two-dimensional image used to quantify polypeptides shown in *A*.

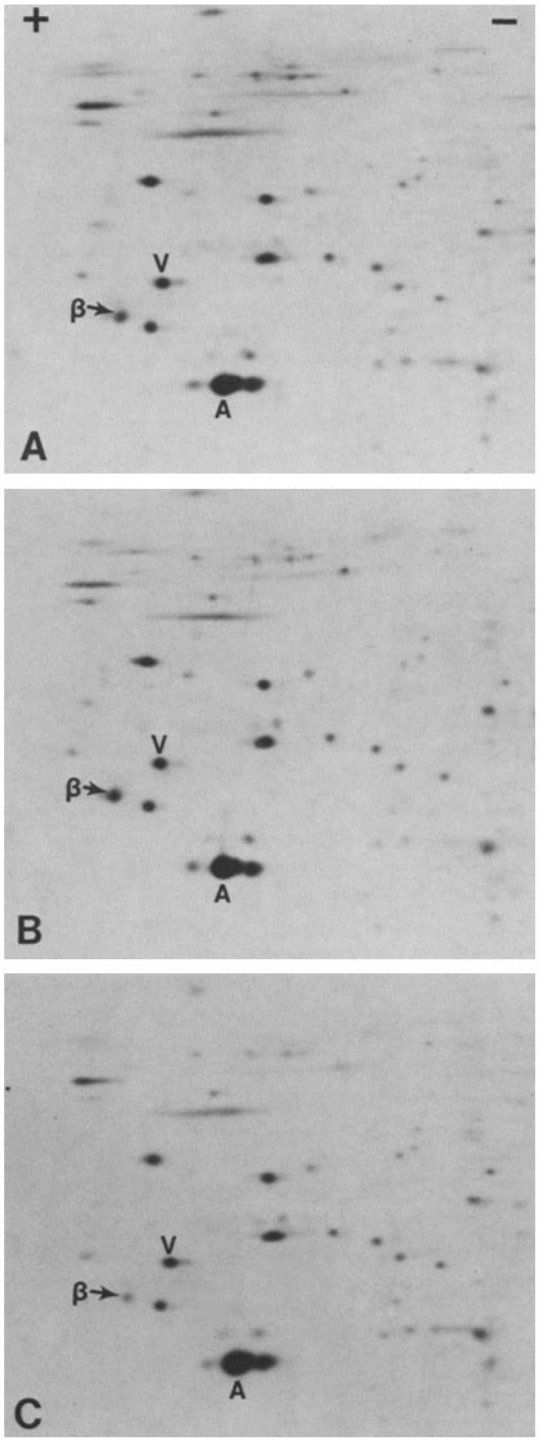

FIGURE 7 Response of cytoplasts to treatment with additional microtubule inhibitors. The pattern of protein synthesis in cytoplasts was determined by two-dimensional gel analysis of pulse-labeled proteins from (*A*) control cytoplasts, (*B*) cytoplasts incubated for 6 h in 10 μM taxol, or (*C*) cytoplasts incubated for 6 h in 10 μM nocodazole. A fluorograph of the resultant patterns is shown. *A*, actin; *β*,-tubulin; *V*, vimentin.

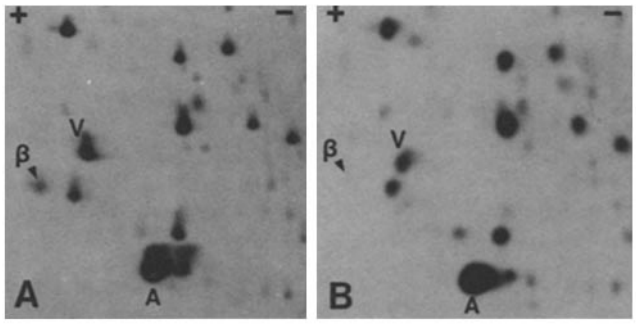

FIGURE 8 Pattern of protein synthesis of individually selected cytoplasts after microtubule depolymerization induced by colchicine. After pulse labeling with [^{35}S]methionine, ~200 cytoplasts were collected by aspiration using a blunt-tipped glass needle attached to a micromanipulator. The cytoplasts were then expelled into O'Farrell lysis buffer (17) and the labeled proteins were resolved by two-dimensional gel electrophoresis. A fluorograph of the resultant patterns is shown. (*A*) Pattern of protein synthesis in control cytoplasts; (*B*) pattern of protein synthesis in cytoplasts treated with 6 h with colchicine. *A*, actin; *β*, *β*-tubulin; *V*, vimentin.

experimental approaches have attempted to identify the molecular events responsible for this regulatory process. Initially, based upon the rough estimation of a 2-h half life of tubulin mRNAs in cells in which all new RNA synthesis was inhibited with actinomycin D, Ben Ze'ev et al. (1) proposed that such regulation was modulated by altered rates of formation of new tubulin RNAs, an hypothesis for which additional indirect support was forthcoming in other experiments using actinomycin D (3). However, serious question of the contribution of transcriptional regulation was raised by measurement of tubulin transcription rates in nuclei isolated from control or colchicine-treated cells. These experiments failed to find any detectable difference in tubulin gene transcription levels (2). By preparing populations of enucleated cell fragments we have now demonstrated that most, if not all of the cellular machinery responsible for establishing *β*-tubulin synthetic rates is retained in enucleated cells. Since we have also shown that the bulk of the proteins synthesized in these cytoplast preparations derive from cytoplasts and not from the small proportion of remaining nucleated cells (Fig. 5 and 8), it is clear that the original hypothesis of transcriptional regulation, which was based upon use of the pleotropic drug actinomycin D, is incorrect.

At first thought, it may be somewhat surprising that cytoplasts can retain such a regulatory mechanism. However, it is well known that cytoplasts are remarkably viable. For example, Albrecht-Buehler demonstrated that even very tiny fragments of enucleated cells remain alive for at least 8 h, as judged by ability to produce and move filopodia or ruffle membranes (20). Similarly, Karsenti et al. (19) noted that regrown microtubule arrays remained intact and apparently normal for as long as 18 h after enucleation. With this in mind, what may be more striking is that *β*-tubulin regulation appears, if anything, to be more complete in the cytoplasts than in whole cells (compare data of Fig. 6 with those of Fig. 1 or with those of Fig. 2 of reference 4). Of course, this finding is expected for a cytoplasmic regulatory event that modulates tubulin mRNA stability: in the absence of a nuclear contribution of newly synthesized *β*-tubulin RNAs, a reduction in cytoplasmic stability will yield a more rapid loss of cytoplasmic RNA content.

However, given the results for *β*-tubulin, the only modest cytoplasmic regulation of new *α*-tubulin synthesis is surprising. Either *α*-tubulin synthesis is not wholly controlled by a cytoplasmic mechanism similar to that for *β*-tubulin or that regulatory machinery is not completely maintained in cytoplasts.

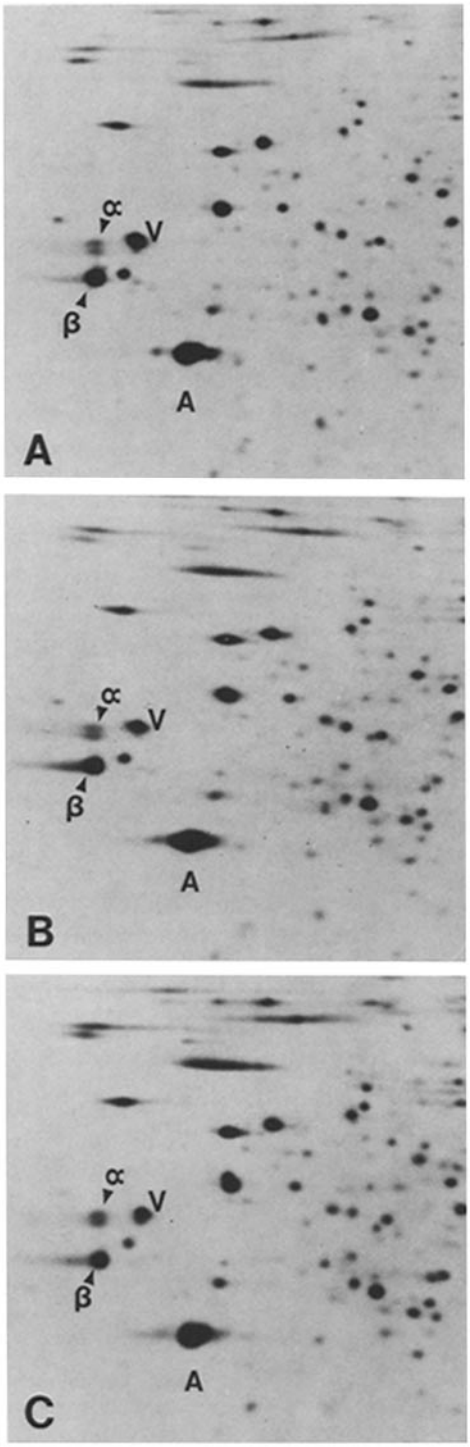

FIGURE 9 Determination of the stability of tubulin polypeptides in cytoplasts. CHO cells were seeded onto each of three coverslips and the cells were labeled with [^{35}S]methionine for 18 h (in methionine-free Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and with one-tenth the normal level of unlabeled methionine). Cytoplasts were then prepared from each slip, and the pattern of prelabeled polypeptides was examined by two-dimensional gel electrophoresis. Shown are the patterns of prelabeled polypeptides in (*A*) cytoplasts examined immediately after enucleation, (*B*) control cytoplasts examined 6 h after enucleation, and (*C*) cytoplasts incubated for 6 h with colchicine. *A*, actin; *β*, β-tubulin; *α*, α-tubulin; *V*, vimentin.

Cytoplasmic control of gene expression has been documented for a variety of previously investigated systems, including the programs of tubulin gene expression in lower eucaryotes. Specifically, Baker et al. (21) have clearly demonstrated that the programmed burst of tubulin synthesis in response to deflagellation in the algae *Chlamydomonas reinhardi* results from a combination of transient transcriptional enhancement and altered tubulin mRNA stability. Similarly, Schedl et al. (22) have demonstrated that tubulin expression in the synchronous cell cycle of *Physarum* is achieved in part by specific changes in tubulin mRNA stability. In addition, in cultured animal cells α-tubulin synthesis has been shown to be modulated apparently through a nontranscriptional mechanism during differentiation of a teratocarcinoma cell line (23). What distinguishes the present example from these others is the intriguing autoregulatory nature of the control event. Although it is not unexpected that such autoregulatory pathways must exist, very few other eucaryotic examples have yet been identified. Moreover, in the best studied of these (T antigen encoded by SV40 [24] and the 70-kD heat-shock protein [25]), the principal level of regulation has been documented to be at the transcriptional level. Hence, cytoplasmic control of apparent autoregulation of tubulin synthesis represents a relatively novel, if not altogether unexpected, pathway for control of expression of a eucaryotic gene.

Moreover, in conjunction with a variety of previous results, the present data have also made it clear that the alteration in tubulin synthetic rates does not simply reflect an inherent cell cycle–dependent program of tubulin synthesis that is uncovered by drug-induced cell cycle disruption. This conclusion arises from four independent lines of evidence. First, although cell cycle control must be severely disrupted in cytoplasts, regulation of β-tubulin synthesis is maintained. Second, even in whole cells the maximum effect is seen within 3 h of drug addition, a time too short to yield a substantial cell cycle blockage in an initially unsynchronized culture. Third, colchicine and nocadozole induce mitotically blocked cell populations that are essentially identical to that induced by vinblastine, even though the effects on tubulin synthetic levels are of opposite signs. Fourth, in contrast to the dramatic repression of tubulin synthesis induced by colchicine or nocodazole treatment, a simple calculation using the reported threefold decline in the overall rate of protein synthesis during mitosis (26) coupled with the known twofold relative increase in tubulin synthesis at mitosis (27) yields a predicted 6% increase in the relative tubulin synthetic rate after a 3-h drug-induced mitotic arrest for cells with a doubling time of 16 h.

But to what physiological end is this cytoplasmic mechanism that regulates tubulin synthesis in an apparent, autoregulatory manner used? Although there are as yet no experimental data that directly approach this most important question, it is clear that intracellular tubulin polymer levels are tied to the subunit levels in some complicated way that involves microtubule initiation sites and microtubule-associated proteins. More specifically, as first described by Kirschner (28), if the free subunit concentration is maintained between C_c^+ (the critical concentration for growth off of a nucleated microtubule whose free end is distal to the centrosome) and C_c^s (the subunit concentration at steady state with a microtubule with both ends free), then nonnucleated polymers will be unstable at the same time that nucleated polymers continue to assemble. Added to this complexity is the potential for

dynamic instability of microtubules as described by Mitchison and Kirschner (29, 30). As a result of this phenomenon even a population of nucleated microtubules may consist of polymers that are slowly elongating at the same time that others in the same population are rapidly disassembling. Consideration of all of these potential effects suggests that even though the assembly process could be self-buffering with a final steady state level of subunits of C_c^+ for a system with nucleated polymers or C_c^s for a system without nucleated polymers, autogenous regulation of tubulin subunit levels may act to augment or even to bypass this control. (Indeed, there is no direct evidence that microtubules are in fact at steady state with subunit levels in vivo.) In any event, given the complexity of the overall assembly process, it seems very likely that in concert with other regulatory events strict autoregulatory control of new tubulin synthesis is used to control carefully the extent and pattern of in vivo microtubule assembly.

We thank Dr. J. Pachter for many stimulating discussions and for communicating his unpublished results.

This work has been supported by grant 83-751 from the American Heart Association and grant GM29513 from the National Institutes of Health (NIH) to D. W. Cleveland, who is also the recipient of an NIH Research Career Development Award.

Received for publication 3 June 1985, and in revised form 5 August 1985.

REFERENCES

1. Ben-Ze'ev, A., S. R. Farmer, and S. Penman. 1979. Mechanisms of regulating tubulin synthesis in cultured mammalian cells. *Cell.* 17:319–325.
2. Cleveland, D. W., and J. C. Havercroft. 1983. Is apparent autoregulatory control of tubulin synthesis nontranscriptionally regulated? *J. Cell Biol.* 97:919–924.
3. Cleveland, D. W., and M. W. Kirschner. 1982. Autoregulatory control of expression of α and β tubulin: implications for microtubule assembly. *Cold Spring Harbor Symp. Quant. Biol.* 46:171–183.
4. Cleveland, D. W., M. A. Lopata, P. Sherline, and M. W. Kirschner. 1981. Unpolymerized tubulin modulates the level of tubulin mRNAs. *Cell.* 25:537–546.
5. Cleveland, D. W., M. F. Pittenger, and J. R. Feramisco. 1983. Elevation of tubulin levels by microinjection suppresses new tubulin synthesis. *Nature (Lond.).* 305:738–740.
6. Cleveland, D. W., M. A. Lopata, and M. F. Pittenger. 1983. Autoregulatory control of the expression of α and β tubulin. *J. Submicrosc. Cytol.* 15:353–358.
7. Taylor, E. W. 1965. The mechanism of colchicine inhibition of mitosis. I. Kinetics of inhibition and the binding of ^{3}H-colchicine. *J. Cell Biol.* 25:145–160.
8. Debrabander, M. J., R. M. L. Van de Veire, F. E. M. Aerts, M. Borgers, and P. A. J. Janssen. 1976. The effects of methyl [5-(2-thienylcarbonyl)-1H-benzimicazol-2-yl carbamate (R 17 934: NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured *in vitro*. *Cancer Res.* 36:905–916.
9. Hiller, G., and K. Weber. 1978. Radioimmunoassay for tubulin: a quantitative comparison of the tubulin content of different established tissue culture cells and tissues. *Cell.* 14:795–804.
10. Spiegelman, B. M., S. M. Penningroth, and M. W. Kirschner. 1977. Turnover of tubulin and the N site GTP in Chinese hamster ovary cells. *Cell.* 12:239–252.
11. Bryan, J. 1971. Vinblastine and microtubules. I. Induction and isolation of crystals from sea urchin oocytes. *Biochemistry.* 11:2611–2616.
12. Schiff, P. B., and S. B. Horwitz, 1980. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA.* 77:1561–1565.
13. Schiff, P. B., J. Fant, and S. B. Horwitz. 1979. Promotion of microtubule assembly in vitro by taxol. *Nature (Lond.).* 277:665–667.
14. Lau, J. T. Y., M. F. Pittenger, J. C. Havercroft, and D. W. Cleveland, 1985. Reconstruction of tubulin gene regulation in cultured mammalian cells. *Ann. NY Acad. Sci.* In press.
15. Prescott, D. M., D. Myerson, and J. Wallace. 1972. Enucleation of mammalian cells with cytochalasin B. *Exp. Cell Res.* 71:480–485.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
17. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
18. Bonner, W. M., and R. A. Laskey. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
19. Karsenti, E., S. Kobayashi, T. Mitchison, and M. Kirschner. 1984. Role of the centrosome in organizing the interphase microtubule array: properties of cytoplasts containing or lacking centrosomes. *J. Cell Biol.* 98:1763–1776.
20. Albrecht-Buehler, G. 1980. Autonomous movement of cytoplasmic fragments. *Proc. Natl. Acad. Sci. USA.* 77:6639–6643.
21. Baker, E. J., J. A. Schloss, and J. L. Rosenbaum. 1984. Rapid changes in tubulin RNA synthesis and stability induced by deflagellation in *Chlamydomonas*. *J. Cell Biol.* 99:2074–2081.
22. Schedl, T., T. G. Burland, K. Gull, and W. F. Dove. 1984. Cell cycle regulation of tubulin RNA level, tubulin synthesis, and assembly of microtubules in Physarum. *J. Cell. Biol.* 99:155–165.
23. Howe, C. C., D. K. Lugg, and G. C. Overton. 1984. Post-transcriptional regulation of the abundance of mRNAs encoding alpha tubulin and a 94,000 dalton protein in teratocarcinoma-derived stem cells versus differentiated cells. *Mol. Cell. Biol.* 4:2428–2436.
24. Myers, R. M., D. C. Rio, A. K. Robbing, and R. Tjian. 1981. SV40 gene expression is modulated by cooperative binding of T antigen to DNA. *Cell.* 25:373–384.
25. DiDomenico, B. J., G. E. Bugaisky, and S. Lindquist. 1982. The heat shock response is self-regulated at both the transcriptional and post-transcriptional levels. *Cell.* 31:593–603.
26. Fan, H., and S. Penman. 1970. Regulation of protein synthesis in mammalian cells. *J. Mol. Biol.* 50:655–670.
27. Bravo, R., and J. E. Celis. 1980. A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. *J. Cell Biol.* 84:795–802.
28. Kirschner, M. W. 1980. Implication of treadmilling for the stability and polarity of actin and tubulin polymers in vivo. *J. Cell Biol.* 86:338–335.
29. Mitchison, T. J., and M. W. Kirschner. 1984. Microtubule assembly nucleated by isolated centrosomes. *Nature (Lond.).* 312:232–237.
30. Mitchison, T. J., and M. W. Kirschner. 1984. Dynamic instability of microtubule growth. *Nature (Lond.).* 312:237–242.