α-Tubulin Limits Its Own Synthesis: Evidence for a Mechanism Involving Translational Repression

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Abstract. A Chinese hamster α-tubulin cDNA was modified to encode an 11-amino acid carboxyl-terminal extension containing the immunodominant epitope from influenza hemagglutinin antigen (to create HAα1-tubulin) and was cloned into a vector for expression in mammalian cells. 12 stable CHO cell lines expressing this HAα1-tubulin were isolated and characterized. HAα1-tubulin incorporated into all classes of microtubules, assembled to the same extent as the endogenous tubulin, and did not perturb the growth of the cells in which it was expressed. However, overexpression of HAα1-tubulin strongly repressed the synthesis of endogenous α-tubulin while having little or no effect on the synthesis of β-tubulin. Treatment of transfected cells with sodium butyrate to induce even greater expression of HAα1-tubulin led to a further decrease in synthesis of endogenous α-tubulin that was fully reversible upon removal of the inducer. Decreased synthesis of α-tubulin in transfected cells did not result from decreased levels of α-tubulin mRNA, as demonstrated by ribonuclease protection assays. On the other hand, colchicine, a drug previously shown to destabilize the tubulin message, caused a clear reduction in both protein synthesis and mRNA levels for transfected HAα1-tubulin and endogenous α-tubulin, thus indicating that the decreased α-tubulin synthesis observed as a result of HAα1-tubulin overexpression is distinct from the previously described autoregulation of tubulin. The results are consistent with a mechanism in which free α-tubulin inhibits the translation of its own message as a way of ensuring stoichiometric synthesis of α- and β-tubulin.

Microtubules are essential structures that direct vesicle and chromosome movement, maintain the structure and location of the Golgi apparatus, and mediate the extended configuration of the ER in eukaryotic cells. These functions are maintained while microtubules undergo a remarkably rapid restructuring throughout the cell cycle. Indeed, inhibition of this dynamic behavior by drug treatment disrupts microtubule function and is cytotoxic (Dhamodharan et al., 1995; Jordan et al., 1993). Since microtubule assembly is concentration dependent, cells have had to acquire mechanisms to regulate tubulin synthesis to arrive at a level of polymer that is appropriate for carrying out microtubule-mediated functions. These include transcriptional mechanisms that determine the pattern of tubulin gene expression in various tissues (Sullivan, 1988), as well as posttranscriptional mechanisms that may act to adjust tubulin synthesis in response to alterations in microtubule assembly. The best known of these latter mechanisms is the autoregulation of tubulin that occurs when animal cells are treated with agents that disrupt or enhance assembly (Cleveland, 1989). For example, treatment with colchicine, an agent that inhibits polymerization of microtubules, decreases the synthesis of both α- and β-tubulin by enhancing the degradation of their respective mRNAs. On the other hand, taxol, a drug that promotes microtubule assembly, stimulates tubulin synthesis and stabilizes tubulin mRNA. It has been suggested that this autoregulatory control is used by cells to maintain constant levels of tubulin heterodimers for assembly (Theodorakis and Cleveland, 1992).

In addition to regulating total tubulin production, cells must also ensure equivalent amounts of α- and β-tubulin subunits to provide the αβ heterodimers necessary for assembly. Previous studies have demonstrated an increased degradation of β-tubulin in mammalian cells when the gene copy number is increased (Gonzalez-Garay and Cabral, 1995; Sisodia et al., 1990; Whitfield et al., 1986), but beyond this, little is known about how cells arrive at equal amounts of α- and β-tubulin. An indication that additional mechanisms beyond degradation of excess subunits are at play has come from recent studies in which an epitope-tagged β-tubulin was overexpressed in CHO cells (Gonzalez-Garay and Cabral, 1995). In response to the increased production of β-tubulin from the transgene, endogenous β-tubulin synthesis decreased by a small amount, while α-tubulin synthesis increased ~25–30%. This suggested the existence of a cellular mechanism whose role is to...
equalize the synthesis of α- and β-tubulin polypeptides (Gonzalez-Garay and Cabral, 1995). In this communication, we describe the response of endogenous tubulin synthesis to increased α-tubulin production. Unlike the results with β-tubulin, where overexpression has only a small effect on its own synthesis, endogenous α-tubulin synthesis is markedly repressed by the introduction of additional α-tubulin genes. The results suggest a mechanism for coordinate production of α- and β-tubulin that operates through the translational repression of α-tubulin synthesis.

Materials and Methods

Construction of a Gene Encoding Epitope-tagged α-Tubulin

We modified the plasmid pRC/HAe1 (Gonzalez-Garay and Cabral, 1995) by adding an EcoRI restriction site before the first codon of the epitope tag using the Transformer II site-directed mutagenesis system (Clontech Laboratories, Inc., Palo Alto, CA). The new plasmid, pRC/HAe1, contains the entire coding sequence for β-tubulin, an EcoRI restriction site at the coding sequence for the epitope tag (hemagglutinin antigen [HA]), and a 3' untranslated sequence derived from SV-40.

The cDNA α1, representing the most abundant CHO α-tubulin (Elliott et al., 1986), was subcloned into the plasmid Bluescript using the restriction enzymes BstEI and HindIII, thereby creating the recombinant plasmid Blαα1. An EcoRI restriction site was then added in front of the termination codon as described above. A HindIII-EcoRI fragment containing the full α1-tubulin coding sequence was gel purified and used to replace the HindIII-EcoRI fragment in pRC/HAe1, thus creating the new plasmid pRC/HAeα1 (Fig. 1).

Isolation of Stable Transfected Cell Lines

DNA was transfected into the wild-type CHO strain 10001 using calcium phosphate precipitation (Chen and Okayama, 1987). Briefly, 20 μg of pRC/HAα1 and 20 μg of herring sperm DNA (Sigma Chemical Co., St. Louis, MO) in 1 ml PBS buffer (25 mM N,N-bis[2-hydroxyethyl]2-aminoethanesulfonic acid, 140 mM NaCl, 125 mM CaCl2, 0.75 mM Na2HPO4, pH 6.95) were added to a 100-mm dish containing 2 x 105 cells in 10 ml of normal growth medium. Two sterile coverslips were placed into the same medium containing 2 μg/ml G-418 (GIBCO BRL) to promote resistance, and resistant colonies were isolated.

Growth and Labeling of Cells

Wild-type CHO cells were maintained at 37°C, 5% CO2 in α-MEM (GIBCO BRL) containing 5% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from GIBCO BRL). Transfected cell lines were grown in the same medium containing 2 μg/ml G-418 (GIBCO BRL) to promote retention of the transfected DNA. In some cases, increased expression of the transfected gene could be induced by treating the culture with 2 mM sodium butyrate, lysing them with hot (100°C) SDS sample buffer (0.0625 M Tris HCl [pH 6.8], 2.5% SDS, 5% 2-mercaptoethanol, 10% glycerol), running the cellular proteins on a 7.5% polyacrylamide minigel (Bio Rad Laboratories, Hercules, CA), and electroblotting and affinity-transferring the proteins onto nitrocellulose (Towbin et al., 1979). The transferred proteins were stained with Ponceau S (Sigma) to identify the position of molecular weight standards and to verify the amount of protein transferred in each lane. The nitrocellulose paper was then blocked by incubation in TTBS (0.05% Tween 20 in PBS) three times for 10 min each. This was followed by incubation in a 1:5,000 dilution of α-tubulin-specific mouse mAb DM 1A (Sigma) for 1 h at room temperature. In some experiments, a 1:5,000 dilution of actin-specific mAb C4 (ICN) was also included to act as an internal control for the amount of protein in each lane. The blot was then washed three times (10 min each) in TTBS and incubated for 1 h in a 1:5,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA). After five more washes (10 min each) in PBS to remove unbound secondary antibody, a peroxidase-catalyzed color reaction product was developed in 1 mg/ml diaminobenzidine (Sigma) in PBS by the addition of 30% H2O2 to a final concentration of 0.03%. Alternatively, detection with a chemiluminescence substrate was carried out according to the manufacturer's instructions (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

Samples for two-dimensional gel electrophoresis were dissolved in MTS buffer (10 mM Tris HCl, pH 6.8, 0.5% NP-40, 0.14 M NaCl, 1 mM MgCl2, 2 mM EGTA), acetone precipitated, and run on two-dimensional gels as described previously (Cabral and Gottesman, 1978; Cabral and Schatz, 1979). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250, dried, and exposed to x-ray film (Hyperfilm MP; Amersham Corp.). In some cases, the proteins resolved by two-dimensional analysis were electrophoretically transferred onto nitrocellulose for subsequent immunodetection with antibodies to tubulin or the HA tag.

Immuno-fluorescence

Cells grown on glass coverslips were left untreated or were treated for 1 h in 0.3 μg/ml Colcemid (Sigma) to depolymerize the microtubules. The cells were then lysed at 4°C for 3 min in MTS buffer without NaCl but containing 4 μg/ml taxol, and fixed in methanol at −20°C at least 6 min. The fixed cells were rehydrated in PBS, and then incubated 1 h at 37°C in PBS containing a 1:10 dilution of mouse mAb DM 1A (Sigma) and a 1:20 dilution of rabbit polyclonal antibody HA.11 (Berkeley Antibody Co., Richmond, CA) specific for the HA tag. After three washes in PBS (5 min each), the cells were incubated for an additional hour in 1:20 dilutions of affinity-conjugated and affinity-purified goat anti-mouse IgG and fluorescein-conjugated and affinity-purified goat anti-rabbit IgG (Kirkegaard & Perry). After three more washes in PBS, the coverslips were inverted onto a small drop (~5 μl) of mounting fluid (90% glycerol, 10% PBS, 0.1% p-phenylenediamine, pH adjusted to 8.6 with KOH), and viewed by epifluorescence using an Optiphoto microscope (Nikon Inc., Melville, NY) equipped with a Plan apochromat ×60 1.4 numerical aperture oil objective (Nikon Inc.) and filter cubes optimized to minimize cross talk between the fluorescein and rhodamine channels (Sawada and Cabral, 1989).

Measurement of Tubulin Synthesis

Cells grown to 70% confluence in 24-well dishes were rinsed two times with PBS and labeled for 1 h in [35S]methionine, as described above. The cells were then washed with PBS, lysed in 100 μl MTS buffer, and centrifuged at 12,000 g. This procedure has been previously shown to release at least 95% of the tubulin into the supernatant fraction (Minotti et al., 1991). Quantitation of the newly synthesized tubulin in the supernatant was carried out by mixing in a known amount of [3H]methionine-labeled CHO cell extract, running the sample on two-dimensional gels, cutting out the tubulin and actin spots, and measuring the [35S]/[3H] ratio for each protein by liquid scintillation counting as described previously (Gonzalez-Garay and Cabral, 1995). To determine the effect of colchicine on tubulin synthesis, cells were treated for 5 h in 0.3 μg/ml colchicine, and then pulse labeled for 1 h in [35S]methionine.
Measurement of Steady-state Tubulin

Relative steady-state levels of tubulin were measured as described for synthesis, except that the cells were metabolically labeled 24 h (two generations) with [3H][35S]methionine before lysis with MTS buffer, and a constant amount of [35S]methionine-labeled cell extract was added to each supernatant as the internal control. The relative amount of tubulin at steady state was calculated from T3H/35S ratios as described previously (Boggs and TX). The cells were scraped into this solution, transferred to a 12,000 g for 2 min. The pellet was rinsed with 80% ethanol, air dried, and resolubilized in diethyl pyrocarbonate treated water. The remaining glass beads were then eliminated by centrifugation.

Measurement of Polymerization

The extent of tubulin polymerization was measured by lysing cells in MTS buffer containing 4 μg/ml taxol and separating the polymerized and soluble tubulin by centrifugation at 12,000 g. Pellet and supernatant fractions were dissolved in SDS sample buffer, resolved on a 7.5% polyacrylamide SDS minigel (BioRad), and the tubulin was immunologically detected on Western transfers as described above. Quantitation was carried out by scanning multiple x-ray film exposures of the blot using a desktop scanner and NIH Image software as described previously (Barlow et al., 1994). The results obtained with this method were confirmed using a more laborious metabolic labeling and two-dimensional gel technique described previously (Minotti et al., 1991). Similar results were obtained by both methods.

Isolation of RNA

Cells were grown in 100-mm dishes to ~80% confluence, the medium was removed, and 2 ml of Ultraspere-B (Biotec Laboratories, Inc., Houston, TX) was added. The cells were scraped into this solution, transferred to a 12,000 g for 15 min. The upper phase was collected, mixed with 0.5 vol of isopropanol and 0.05 vol of RNAtrack resin (Biotec), and centrifuged at 12,000 g for 15 min. The upper phase was collected mixed, with 0.5 vol of isopropanol and 0.05 vol of RNAtrack resin (Biotec), and centrifuged at 12,000 g for 2 min. The pellet was rinsed with 80% ethanol, air dried, and resolubilized in diethyl pyrocarbonate-treated water. The remaining glass beads were then eliminated by centrifugation.

RNAse Protection Assay

A labeled antisense riboprobe that overlaps sequences from α1-tubulin and the HA tag was generated by linearizing the plasmid pRC/HAα1 with the restriction enzyme C131 and using it as template for SP6 RNA polymerase in the presence of [,α-32P]UTP. For internal controls, a DdeI linearized fragment of mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Ambion, Inc., Austin, TX) transcribed with SP6 or a fragment of γ-actin (Nagy et al., 1995) were used. The resulting riboprobes were gel purified and used in the RNAse protection (RNPase) analysis. To measure relative levels of mRNA for endogenous α-tubulin, HAα1-tubulin, and GAPDH or γ-actin, 10 μg of total RNA from control and transfected cell lines were hybridized in solution (40 mM Pipes, pH 6.7, 0.4 M NaCl, 1 mM EDTA, and 80% formamide) with the 32P-labeled RNA probes described above, and the resulting RNA-RNA hybrids were digested in RNase digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA and 300 mM NaCl) with RNase A and T1 for 3 h at 37°C. The digestion was stopped by the addition of SDS and proteinase K, and the protected fragments were resolved on a 6% denaturing polyacrylamide gel. The dried gel was exposed to x-ray film to obtain an image, but was quantitated by placing the band and measuring radioactivity in a liquid scintillation counter (model LS2000; Beckman Instruments, Inc., Fullerton, CA).

Results

Isolation of Stable Cell Lines Expressing HAα1-Tubulin

To begin assessing the effects of α-tubulin overproduction on the synthesis of the endogenous tubulin polypeptides, an epitope-tagged α-tubulin gene was constructed to encode α-tubulin with altered immunological and electrophoretic properties. Since previous experience had already shown that a nine-amino acid epitope from influenza HA placed at the carboxyl terminus of β-tubulin produced a polypeptide with normal assembly properties in vivo (Gonzalez-Garay and Cabral, 1995), a sequence encoding the same epitope tag was introduced after the last codon of CHO α1, a cDNA representing the most abundant α-tubulin isotype expressed in CHO cells (Elliott et al., 1986). To facilitate construction of this gene and its integration into a pRC/CMV expression plasmid, an Eco47III site was engineered at the TAA termination codon resulting in the insertion of an additional two amino acids (Ser Ala). This allowed a simple swap of the α-tubulin coding sequence for the β-tubulin coding sequence in pRC/HAβ1, which was modified to have a similar Eco47III site immediately before the epitope-tagging sequence (Gonzalez-Garay and Cabral, 1995). The resulting plasmid, pRC/HAα1 (Fig. 1), contains 56 nucleotides of upstream 5’ untranslated sequence, the entire α1-tubulin coding sequence, the epitope tag sequence at the 3’ end of the gene, and 3’ untranslated sequences derived from SV-40.

The pRC/HAα1 plasmid was transfected into wild-type CHO cells and stable cell lines were selected using the neomycin analogue, G418. A total of 18 resistant colonies were isolated and, of these, 12 were positive for expression of HAα1-tubulin, a frequency similar to that obtained with transfected HAβ1-tubulin and transfected microtubule-associated proteins in previous studies (Barlow et al., 1994; Gonzalez-Garay and Cabral, 1995). Extracts from each of the G418-resistant cell lines were resolved by SDS-PAGE, transferred onto nitrocellulose, and stained with DM 1A, a liquid scintillation counter (model LS2000; Beckman Instruments, Inc., Fullerton, CA).

Gonzalez-Garay and Cabral Translational Regulation of α-Tubulin 1527
HAα1-Tubulin Exhibits Normal In Vivo Behavior

To determine whether HAα1-tubulin assembled into normal microtubule structures, transfected cells were examined by immunofluorescence after double staining with the α-tubulin-specific mouse mAb DM 1A and with HA.11, a rabbit polyclonal antibody specific for the epitope tag. The epitope-tagged α-tubulin was found to assemble into all cytoplasmic microtubules (Fig. 3, A and B), as well as into spindle microtubules (Fig. 3, A and B, insets). Furthermore, pretreatment of cells with Colcemid indicated that the microtubules remained drug sensitive and that HAα1-tubulin (upper band) and endogenous α-tubulin (lower band) were detected. The relative intensity of the upper band was increased in each case by sodium butyrate treatment. The numbers indicate independently isolated clones, and c indicates a cell extract from PRC, a cell line transfected with vector alone.

A more quantitative estimate for the ability of HAα1-tubulin to assemble was obtained by comparing the ability of endogenous α-tubulin and transfected HAα1-tubulin to fractionate into cytoskeletal and soluble intracellular pools before and after drug treatment. For these experiments, cells were lysed with a microtubule-stabilizing buffer containing taxol, tubulin was separated into soluble and cytoskeletal fractions by centrifugation, the proteins in each fraction were resolved by SDS gel electrophoresis, and the fraction was 38% of the total, a value similar to one that we have previously reported for the assembly of tubulin in wild-type CHO cells (Minotti et al., 1991). An independent measure of HAα1-tubulin assembly into microtubules was obtained using a sensitive two-dimensional gel based assay that is capable of measuring small changes in assembly in mutant cell lines (Minotti et al., 1991). In agreement with the results of the Western blot assay, it was found that 39% of HAα1-tubulin appeared in the cytoskeletal fraction. It thus appears that HAα1-tubulin does not perturb the extent of microtubule assembly in the cells where it is expressed.

Expression of HAα1-Tubulin Represses the Synthesis of Endogenous α-Tubulin

Previous studies from this laboratory have shown that overexpression of transfected HAβ1-tubulin in CHO cells results in a small decrease (to 84% of normal) in endogenous β-tubulin synthesis and a larger increase (to 129% of normal) in α-tubulin synthesis (Gonzalez-Garay and Cabral, 1995). The changes suggested the existence of a mechanism that acts to maintain coordinate syntheses of α- and β-tubulin. One possible prediction from these earlier studies was that overexpression of transfected HAα1-tubulin might lead to enhanced synthesis of endogenous β-tubulin. To test this possibility, stable cell lines transfected with HAα1-tubulin (e.g., clone 9) or with the vector alone (PRC) were pulse-labeled with [35S]methionine, and cell extracts were resolved by two-dimensional gel electrophoresis. To incorporate sufficient counts for subsequent quantitation, we used a 60-min labeling time to measure synthesis; however, the possibility existed that incorporation of counts during the 60 min might reflect not only synthesis, but also potentially rapid degradation of any α-tubulin that might be displaced from heterodimers by the overexpressed HAα1-tubulin. We therefore also examined labeling times as short as 10 min and obtained virtually identical results, indicating that tubulin turnover is not rapid enough to significantly affect synthesis rates measured in a 60-min pulse. Autoradiograms from a few of the gels actually used for quantitation are shown in Fig. 5. Comparison of clone 9 (Fig. 5 B) with PRC (Fig. 5 A) indicates the presence of new spots (large arrow) migrating more slowly and at a more acidic isoelectric point than endogenous α-tubulin (small arrow) in the transfected cells. These new spots have the expected mobility for HA-tagged α-tubulin, and their identity was confirmed by immunostaining with antibodies specific for α-tubulin and for the HA tag (data not shown). Comparison of endogenous α- and β-tubulin in clone 9 and PRC indicates that expression of HAα1-tubulin in the transfected cells results in less α-tubulin synthesis, but has little or no effect on β-tubulin synthesis (Fig. 5, A and B). The decrease in endogenous α-tubulin synthesis is magnified when expression of HAα1-tubulin is further enhanced by treating the cells with sodium butyrate (Fig. 5 C). After induction, there appears to be very little synthesis of endogenous α-tubulin but essentially normal synthesis of β-tubulin in the transfected cells.

Changes in tubulin synthesis and steady-state levels in transfected cells were quantitated by measuring the radio-
activity in tubulin spots excised from two-dimensional gels, and the results are summarized in Table I. As predicted from visual examination of the autoradiograms (e.g., Fig. 5), production of HAα1-tubulin in clone 9 caused a significant reduction (to 61% of normal) in the synthesis of endogenous α-tubulin while having little or no effect on the synthesis of β-tubulin. When the same transfected cells were treated with sodium butyrate to induce further expression of HAα1-tubulin, an even greater decrease (to 34% of normal) in endogenous α-tubulin synthesis was measured, but there was still no effect on β-tubulin synthesis. It should be pointed out that these values probably underestimate the actual decrease in α-tubulin synthesis because of problems associated with determining the true background correction for spots excised from two-dimensional gels. This problem is especially evident in butyrate-treated cells, where the synthesis of α-tubulin falls to very low levels and the errors associated with background correction become more prominent. Thus, the visual perception for the magnitude of the decrease on autoradiograms at all exposures is usually greater than the actual measurements. The decreases in endogenous α-tubulin synthesis were more than compensated by the production of HAα1-tubulin so that the total amount of α-tubulin synthesis (endogenous plus transfected) was ~139% of normal in untreated cells and 428% of normal after overnight treatment with 2 mM sodium butyrate.

In addition to elevated total α-tubulin synthesis in transfected cells, the amount accumulated at steady state also increased, but to a lesser extent than predicted from the synthesis rates (Table I). This probably reflects some enhanced degradation of α-tubulin that is not paired with β-tubulin. Although similar results have been reported in yeast (Burke et al., 1989; Katz et al., 1990), this is the first direct evidence for enhanced degradation of free α-tubulin in mammalian cells. The agreement between steady-state level and synthesis appeared to be closer for endogenous α-tubulin than for HAα1-tubulin, indicating that HAα1-tubulin may be preferentially degraded. Even though much of the excess α-tubulin is degraded, the increase in steady-state levels suggests that α-tubulin is synthesized faster than it can be cleared by degradation. Since there is no evidence for an increased accumulation of β-tubulin, the results suggest that a small pool of free α-tubulin may exist in the transfected cells.

Repressed Synthesis of α-Tubulin Is Reversible

To determine how rapidly changes in α-tubulin synthesis are seen, one of the HAα1-tubulin–transfected cell lines

![Figure 3. Immunofluorescence of clone 9, a cell line with stable expression of HAα1-tubulin. Cells were permeabilized in MTS buffer containing taxol, fixed in methanol, and simultaneously stained with mouse mAb DM 1A (A and C) and rabbit antibody HA.11 (B and D) that recognizes the epitope tag. In C and D, the cells were pretreated with 0.3 μg/ml Colcemid for 1 h before cell lysis, which led to the disappearance of most cytoplasmic microtubules and allowed better visualization of the microtubule-organizing centers (arrows). The insets in A and B show staining of the mitotic spindle apparatus. Bar, 10 μm.](image-url)
The decreased synthesis of endogenous α-tubulin in HAed-tubulin is reversible. To address this issue, transfected cells were treated with 2 mM sodium butyrate to induce further expression of the transfected gene. At various times after removal of the butyrate, the cells were incubated for 1 h with [35S]methionine to label newly synthesized proteins, and cell extracts were resolved by two-dimensional gel electrophoresis. Autoradiograms of these gels are shown in Fig. 6. A significant decrease in the synthesis of endogenous α-tubulin (small arrow) is seen by 3 h after sodium butyrate addition (Fig. 6 C), and this decrease is already close to maximal by 7 h (Fig. 6 D). Comparison of the synthesis of endogenous α-tubulin with transfected HAα1-tubulin (large arrow) shows a reciprocal relationship: as HAα1-tubulin synthesis increases, endogenous α-tubulin synthesis decreases. The time course of the decrease in endogenous α-tubulin synthesis is consistent with the amount of time needed to increase transcription of the HAα1-tubulin and allow for accumulation of the protein product in the tubulin pool.

The large decrease observed in endogenous α-tubulin synthesis raises the question of whether the change is reversible. To address this issue, transfected cells were treated for 24 h with 2 mM sodium butyrate to reach a new "steady state," the sodium butyrate was washed out, and the cells were again labeled for 1 h with [35S]methionine at various times after removal of the butyrate. Little or no further decrease in the relative synthesis of α-tubulin was noted between 7 and 24 h of sodium butyrate treatment (compare Fig. 6, D and E). By 7 h after the removal of sodium butyrate, however, the relative rates of the syntheses of α- and HAα1-tubulin had returned to normal (Fig. 6 G) and remained normal up to 24 h after the reversal (Fig. 6 H). Thus, the changes in α-tubulin synthesis caused by the expression of transfected HAα1-tubulin appear to be fully reversible.

Repression of α-Tubulin Occurs without a Decrease in Message Levels

The decreased synthesis of endogenous α-tubulin in HAα1-tubulin transfected cells could be caused by decreased transcription of α-tubulin gene(s), enhanced degradation of the α-tubulin mRNA, or decreased translation of the existing message. To determine whether decreased synthesis reflected a decrease in the mRNA encoding α-tubulin, RPAse assays were carried out. A labeled antisense riboprobe that hybridizes 217 nucleotides from the 3' end of the coding sequence for α1-tubulin and an additional 79 nucleotides of epitope tag and 3' untranslated sequences was prepared. Thus, hybridization with HAα1-tubulin mRNA should protect a 296-bp fragment, while hybridization to endogenous α-tubulin mRNA should protect a smaller 217-bp fragment. A second labeled antisense riboprobe that hybridizes to GAPDH was also included to act as an internal control for the amount of RNA in each experiment. The results from one such experiment are shown in Fig. 7. Hybridization of the radiolabeled probes with RNA derived from wild-type or PRC cells led to the protection of two major fragments of 217 and 150 bp char-

**Table 1. Synthesis and Steady-state Levels of Tubulin in HAα1-Tubulin–transfected Cells**

| α-Tubulin | Steady state | Synthesis | Steady state |
|-----------|-------------|-----------|-------------|
| Butyrate  | + Butyrate  | Butyrate  | + Butyrate  |
| α-Tubulin | 61 ± 6%     | 34 ± 10%  | 63 ± 10%    | 29 ± 8%      |
| β-Tubulin | 98 ± 9%     | 104 ± 6%  | 99 ± 3%     | 80 ± 6%      |
| HAα1-tubulin | 78 ± 5% | 394 ± 25% | 65 ± 10%    | 172 ± 8%     |
| Total α** | 139%        | 428%      | 128%        | 201%         |

*aCells* (untreated or pretreated overnight with 2 mM sodium butyrate) were pulse labeled 1 h in [35S]methionine, run on two-dimensional gels, and the amount of tubulin was quantitated by liquid scintillation counting. The tubulin counts were normalized by dividing by the actin counts from the same gel, and the results are given as the percent present in HAα1-tubulin–transfected cells (clone 9) relative to normalized values (arbitrarily set at 100%) for the same species in wild-type cells transfected with vector alone (PRC cells). The control for butyrate-treated clone 9 was PRC cells treated in parallel with the same concentration of butyrate (2 mM).

†Endogenous α-tubulin.

‡Endogenous β-tubulin.

§Transfected HAα1-tubulin.

**Total α equals endogenous α plus transfected HAα1-tubulin. Each measurement represents an average of four to seven experiments, each run in triplicate. The average deviation from the mean is given.
characteristic for α-tubulin and GAPDH, respectively (Fig. 7, lanes 1 and 2). In contrast, hybridization with RNA derived from clone 9 cells, which express HAα1-tubulin, produced three major protected fragments—the same two that were protected with the control RNA, as well as a 296-bp fragment characteristic of HAα1-tubulin mRNA (Fig. 7, lane 3). A control in which the probes were hybridized with transfer RNA and digested produced no major protected fragments (Fig. 7, lane 4). Comparison of untreated wild type, PRC, and clone 9 (Fig. 7 A) did not show a specific reduction in the 217-bp band in clone 9, as might be expected from its reduced α-tubulin protein synthesis. The experiment was repeated three times using both GAPDH and γ-actin as internal controls, the bands were quantitated by direct counting, and the results are summarized in Table II. As predicted from visual inspection of Fig. 7, it was found that the level of α-tubulin mRNA in HAα1-tubulin–transfected cells was unchanged relative to wild-type or PRC cells. Furthermore, in cells treated with sodium butyrate, the level of HAα1-tubulin mRNA was clearly increased in clone 9, but again there was no reduction in α-tubulin mRNA compared to wild-type and PRC cells (Fig. 7 C and Table II) despite the fact that α-tubulin synthesis in clone 9 is only 34% of normal under these conditions (see Table I). The results indicate that the reduced synthesis of endogenous α-tubulin observed in HAα1-tubulin–transfected cells is not a consequence of decreased message levels, but must reflect decreased translation of the existing message.

Colchicine Reduces Protein Synthesis and mRNA Levels for Both Endogenous and Transfected α-Tubulin

Previous studies from many laboratories have described a tubulin autoregulatory mechanism in which treatment of...
animal cells with colchicine results in a specific decrease in the synthesis of both α- and β-tubulin that is mediated through increased degradation of their respective mRNAs (for review see Cleveland, 1989). To determine whether the repression of α-tubulin synthesis we are seeing is related to this mechanism or represents a separate pathway, PRC and clone 9 cells were treated with a low concentration (0.3 μg/ml) of colchicine for 5 h before or after maximum expression of HAα1-tubulin was induced with sodium butyrate. After colchicine treatment, protein synthesis was monitored by labeling the cells for 1 h with [35S]methionine, separating cellular proteins by two-dimensional gel electrophoresis, and measuring the radioactivity associated with tubulin and actin as already described. The data summarized in Table III demonstrate that the syntheses of HAα1-tubulin and endogenous α-tubulin are reduced after colchicine treatment, as would be predicted from the tubulin autoregulatory mechanism. When the corresponding levels of α-tubulin mRNA were examined using RPase, a clear decrease in the 217-bp α-tubulin-protected fragment was seen in wild type, PRC, and clone 9 after colchicine treatment in both uninduced (Fig. 7, compare A and B) and sodium butyrate-induced cells (Fig. 7, compare C and D). Quantification of these changes by direct counting of the radioactive associated with each band is summarized in Table III. The data show a clear reduction in both synthesis and mRNA levels in transfected and nontransfected cells after colchicine treatment. This result indicates that we can reliably measure small changes in mRNA levels using the RPase assay, and that the reduction in endogenous α-tubulin synthesis we observe in HAα1-tubulin–transfected cells is not a consequence of the previously described tubulin autoregulatory mechanism, but rather represents a novel form of tubulin regulation.

Table III. Effect of Colchicine on α-Tubulin Protein Synthesis and mRNA levels

|              | PRC | Clones 9 | PRC | Clones 9 |
|--------------|-----|----------|-----|----------|
| α-Tubulin    | 63 ± 7% 38 ± 5% | 60 ± 9% 52 ± 4% | 50 ± 7% 29 ± 6% | 59 ± 10% 46 ± 4% |
| HAα1-tubulin | -   | -        | 50 ± 6% 60 ± 9% | 24 ± 2% 29 ± 2% |

Synthesis was measured as described in the legend to Table I, and mRNA levels were measured by RNase protection. In some cases, the cells were preincubated overnight with 2 mM sodium butyrate. The values given are for tubulin in cells treated for 5 h with 0.3 mg/ml colchicine compared to the same tubulin species in the same cells without colchicine treatment set arbitrarily at 100%. Each value represents the average of three experiments. The average deviation from the mean is given.

Discussion

The forced expression of exogenously supplied genes represents a powerful approach for studying how cells regulate the production of specific proteins. In this communication, we report the effects of overproduction of exogenously supplied α-tubulin on the synthesis of endogenous α-tubulin. Although the transfected gene encoded HAα1-tubulin with an 11-amino acid extension at the carboxyl-terminal end, it was found that this epitope-tagged tubulin assembled normally and did not grossly affect the growth or behavior of the cells in which it was expressed. The presence of the epitope tag did, however, allow the exogenously supplied gene product to be distinguished from the endogenous α-tubulin by immunological and electrophoretic techniques.

Previous studies using this approach with β-tubulin produced evidence for a mechanism that acts to maintain the coordinate syntheses of α- and β-tubulin (Gonzalez-Garay and Cabral, 1995). Although the overexpression of HAβ1-tubulin produced a small decrease (16%) in endogenous β-tubulin synthesis, it also produced a significant increase (29%) in α-tubulin synthesis in the transfected cells. The results suggested that cells were able to react to the increased production of HAβ1-tubulin, and attempted to adjust the syntheses of endogenous α- and β-tubulin in a compensatory manner to maintain coordinate levels of the two subunits. The current studies were undertaken to test whether similar compensatory changes would occur upon overproduction of HAα1-tubulin. We found that in contrast to the results with HAβ1-tubulin, overexpression of HAα1-tubulin produced little or no change in the synthesis of the interacting subunit (β-tubulin). Instead, large decreases in the synthesis of endogenous α-tubulin were found. Together, these two studies indicate that coordinate regulation of α- and β-tubulin syntheses occurs primarily through changes in the synthesis of the α subunit.

A model to explain this mechanism is diagrammed in Fig. 8. Transcription of α- and β-tubulin genes determines the maximum possible rate of tubulin synthesis. Since α- and β-tubulins are encoded by multigene families in vertebrate cells (Sullivan, 1988), certain tissues may increase or decrease intracellular tubulin levels, based on which tubulin genes are switched on. However, other downstream regulatory mechanisms may also help specify the intracellular concentration of tubulin. For example, the amount of message for α- and β-tubulin is determined not only by transcription, but also by message degradation. At least one means by which this can occur involves modulated degradation of mRNA for both α- and β-tubulin that appears to be mediated by the distribution of αβ heterodimers between the soluble and polymer pools (for review see Cleveland, 1989). Another possible way in which tubulin levels might be regulated is through degradation of the protein itself. It is generally accepted that tubulin exists in the form of αβ heterodimers, and these appear to be very stable with half-lives for degradation of >50 h (Spiegelman et al., 1977, and our unpublished observations). Therefore, under normal conditions, protein degradation does not appear to play a major role in fine-tuning intracellular tubulin levels. However, this situation may vary with other conditions in which tubulin is...
damaged by toxins or by the acquisition of mutations (Boggs and Cabral, 1987; Kemphues et al., 1982).

Although degradative mechanisms do not appear to play a large role in maintaining intracellular levels of αβ heterodimers, they may help to ensure that coordinate levels of α- and β-tubulin are maintained for heterodimer formation. There is little evidence to suggest that appreciable levels of free α- or β-tubulin exist in any cell type; and in fact, the rapid degradation of free β-tubulin subunits has been demonstrated in a variety of organisms (Gonzalez-Garay and Cabral, 1995; May et al., 1990; Sisodia et al., 1990; Whitfield et al., 1986). The only known exception appears to be some strains of Saccharomyces cerevisiae, where overproduced β-tubulin has been shown to accumulate as free subunits and produce cytotoxicity (Burke et al., 1989; Katz et al., 1990). Stable cell lines overproducing α-tubulin have not previously been reported in mammalian cells. In our studies, we found, in agreement with studies in yeast (Burke et al., 1989; Katz et al., 1990), that overproduced α-tubulin is rapidly degraded and that significant steady-state levels of free α-tubulin probably do not exist under normal conditions. It thus appears that degradative mechanisms are capable of ridding mammalian cells of large excesses of uncomplexed tubulin subunits that might otherwise have deleterious effects on growth.

Our data also indicate the existence of a novel translational mechanism of tubulin regulation that may act to maintain the coordinate syntheses of α- and β-tubulin. This pathway may exert a finer level of control on relative levels of α- and β-tubulin than the degradative pathway. It is unlikely that the reduced production of endogenous α-tubulin in HAO1-tubulin-transfected cells that we measure in a 1-h pulse can be explained by very rapid degradation of uncomplexed α-tubulin. This conclusion is based on the following observations: (a) we get essentially identical results using pulse-labeling times as short as 10 min (data not shown); (b) if uncomplexed α-tubulin were degraded as rapidly as it is synthesized, we would not expect to be able to measure either the excess synthesis of HAO1-tubulin over endogenous β-tubulin in a 1-h pulse or the increased accumulation of total α-mammalian in transfected cells in a 16-24-h labeling experiment (Table 1); and (c) when HAB1-tubulin is overexpressed, we do not see large decreases in endogenous β-tubulin synthesis using a 1-h pulse, even though uncomplexed β-tubulin appears to be less stable than uncomplexed α-tubulin, based on our inability to measure excess accumulation in a 16–24-h labeling experiment (Gonzalez-Garay and Cabral, 1995). We therefore conclude that cells possess a mechanism that regulates the synthesis of α-tubulin.

The key feature of this mechanism is an autofeedback loop in which monomeric α-tubulin limits its own synthesis. Because mRNA levels for α-tubulin remain high under conditions in which synthesis of the protein is strongly repressed, we suggest that modulation of synthesis is mediated by translational effects. One possible way in which this could occur, and the one that we currently favor, is that monomeric α-tubulin is able to bind to its own message and inhibit the formation of the 40S ribosomal initiation complex or the subsequent scanning of the message to the translational start site (for a review of translational mechanisms see Hershey, 1991). This inhibition may be relieved when α-tubulin binds to the β-subunit, thus allowing the synthesis of α-tubulin to be adjusted to the level of existing β-tubulin subunits. Such a mechanism would explain the increase in α-tubulin synthesis that is seen when β-tubulin is overexpressed (Gonzalez-Garay and Cabral, 1995). In this previous study, the level of α-tubulin synthesis increased by only 25–30%, even when the synthesis of total β-tubulin was increased approximately fivefold over normal by sodium butyrate induction, suggesting that α-tubulin synthesis in CHO cells is already close to its maximum possible rate under normal conditions.

It should be pointed out that while the idea that α-tubulin represses its own synthesis is consistent with the data, we cannot presently rule out the possibility that β-tubulin, alone or in combination with some other factor, binds to α-tubulin mRNA and directly promotes translation. However, there is currently little evidence for this type of translational control, except in viral systems (Standart and Jackson, 1994), and the fact that α-tubulin can be successfully translated in vitro in the absence of new β-tubulin synthesis (Yaffe et al., 1988) leads us to prefer a mechanism in which α-tubulin acts as a repressor of its own translation. Whatever the actual mechanism, our results suggest that α-tubulin synthesis is controlled by the availability of β-tubulin for heterodimer formation.

Other features of the model remain to be studied. For
example, it is not yet clear what region of the α-tubulin message mediates the translational effects. By analogy with other systems in which translational repression has been reported (Klausner et al., 1993; Standart and Jackson, 1994), it is possible that some secondary structure in the 5′ untranslated region (UTR) might be stabilized by binding monomeric α-tubulin and thereby inhibit translation. Alternatively, sequences in the 3′ UTR could influence translational events in the 5′ UTR by interactions promoted through mRNA folding, and this folding could in turn be influenced by the availability of monomeric α-tubulin.

The observation that the synthesis of the transfected HAα1-tubulin is not repressed supports the idea that a critical region for repression must involve the 5′ or 3′ UTRs. Because the transfected HAα1-tubulin gene is derived from CHO and has the identical coding sequence to the endogenous α-tubulin (Elliott et al., 1986), it seems reasonable to conclude that sequences within the coding region are not sufficient to mediate repression. Furthermore, the gene contains 56 nucleotides from the α-tubulin 5′ UTR, making it likely that sequences further upstream are involved if the regulatory element resides in the 5′ UTR.

Although the molecular details remain to be elucidated, this report describes the first translational mechanism for control of coordinate production of α- and β-tubulin. Making the synthesis of one subunit dependent on the availability of a second interacting subunit provides an attractive and economical means for cells to arrive at appropriate concentrations of subunits for the assembly of multisubunit complexes. A precedent for this type of translational control is provided by ribosomal protein synthesis in bacteria (for reviews see Lindahl and Hinnenbush, 1992; Nomura et al., 1984). However, only a small handful of examples where the protein product of a gene inhibits its own translation currently exist in mammalian cells (for review see Standart and Jackson, 1994). Of these, most involve regulation of the levels of an enzyme or binding protein based on the availability of the substrate or a substrate-like inhibitor (Chu et al., 1991, 1993). To our knowledge, this is the first example in eukaryotic cells where translational repression appears to regulate the coordinated synthesis of interacting proteins. We suspect it will not be the last.

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