MOLECULAR AND CELLULAR BIOLOGY, Aug. 1989, p. 3418–3428
0270-7306/89/083418-11$02.00/0

Vol. 9, No. 8

Assembly Properties of Altered β-Tubulin Polypeptides Containing Disrupted Autoregulatory Domains

WEI GU AND NICHOLAS J. COWAN*

Department of Biochemistry, New York University Medical Center, 550 First Avenue, New York, New York 10016

Received 6 December 1988/Accepted 25 April 1989

β-Tubulin synthesis in eucaryotic cells is subject to control by an autoregulatory posttranscriptional mechanism in which the first four amino acids of the β-tubulin polypeptide act either directly or indirectly to control the stability of β-tubulin mRNA. To investigate the contribution of this amino-terminal domain to microtubule assembly and dynamics, we introduced a series of deletions encompassing amino acids 2 to 5 of a single mammalian β-tubulin isotype, Mβ1. Constructs carrying such deletions were inserted into an expression vector, and the ability of the altered polypeptide to coassemble into microtubules was tested by using an anti-Mβ1-specific antibody. We show that the Mβ1 β-tubulin polypeptide was competent for coassembly into microtubules in transient transfection experiments and in stably transfected cell lines when it lacked either amino acid 2 or amino acids 2 and 3. The capacity of these mutant β-tubulins to coassemble into polymerized microtubules was only slightly diminished relative to that of unaltered β-tubulin, and their expression did not influence the viability or growth properties of cell lines carrying these deletions. However, more extensive amino-terminal deletions either severely compromised or abolished the capacity for coassembly. In analogous experiments in which alterations were introduced into the amino-terminal domain of a mammalian α-tubulin isotype, Mα4, deletion of amino acid 2 did not affect the ability of the altered polypeptide to coassemble, although removal of additional amino-terminal residues essentially abolished the capacity for competent coassembly. The stability of the altered assembly-competent α- and β-tubulin polypeptides was measured in pulse-chase experiments and found to be indistinguishable from the stability of the corresponding unaltered polypeptides. An assembly-competent Mα4 polypeptide carrying a deletion encompassing the 12 carboxy-terminal amino acids also had a half-life indistinguishable from that of the wild-type α-tubulin molecule. These data suggest that the universally conserved amino terminus of β-tubulin acts largely in a regulatory role and that the carboxy-terminal domain of α-tubulin is not essential for coassembly in mammalian cells in vivo.

Microtubules are a fundamental component of the cytoskeleton in eucaryotic cells. They are composed principally of two soluble proteins, α- and β-tubulin, which, together with microtubule-associated proteins (MAPs), assemble into 20-nm filamentous polymers. Microtubules perform many functions in eucaryotic cells, including segregation of chromosomes during meiosis and mitosis, determination of cell shape via dynamic modulation of cytoplasmic microtubules, cell motility, as an integral part of cilia and flagella, and bidirectional transport of organelles along the axons of neuronal cells.

An important question regarding the biology of microtubules is the relationship between tubulin primary sequence and the structural organization and functional diversity of microtubules. Our knowledge of the functional contribution of different domains within the tubulin molecule is extremely limited. Recently, however, Cleveland and co-workers have shown that the first four amino acids of β-tubulin (Met, Arg, Glu, and Ile), which are universally conserved among all β-tubulin polypeptides (reviewed in S. A. Lewis and N. J. Cowan, *in* J. Avila, ed., *Microtubule Proteins*, in press), are both necessary and sufficient to confer autoregulated instability of β-tubulin mRNAs (8, 25, 35, 36). In addition, the amino terminus of α-tubulin consists of a sequence in which the first three amino acids are identical to those of the corresponding domain of β-tubulin and are universally conserved among α-tubulin polypeptides (Lewis and Cowan, in press). However, the structural and functional significance of the α-tubulin aminoterminal domain is less certain.

Studies at the molecular level have shown that, within both the α- and β-tubulin multigene families, the encoded carboxy-terminal regions are the most divergent. This and other characteristic differences among tubulin polypeptides serve to define tubulin isotypes (31–33; Lewis and Cowan, in press). Sequences characteristic of each isotype are, with few exceptions, absolutely conserved among mammalian species. In vitro studies have shown that the carboxy-terminal domains of α- and β-tubulin play a significant role in controlling tubulin interactions leading to microtubule assembly (27, 29) and the carboxyterminal domain also contributes to interactions with MAPs (19, 20, 23).

Several questions remain to be answered with regard to the amino- and carboxy-terminal regions of α- and β-tubulins. Though the first four amino acids of β-tubulin are implicated in autoregulation at the mRNA level, it is not clear whether the function of this domain is exclusively regulatory or whether it also contributes to the ability of the subunits to polymerize into microtubules and other physiological properties pertaining to microtubule behavior in vivo. With regard to the carboxy-terminal domain, in vivo experiments have shown that removal of 12 amino acids from the carboxy terminus of the single β-tubulin polypepetide expressed in *Saccharomyces cerevisiae* has no effect on a number of microtubule-dependent functions, such as mitotic and meiotic division and mating under optimal growth conditions (7, 12, 21). In mammals, a chimeric β-tubulin composed of a vertebrate domain fused to a yeast carboxy-terminal domain is competent for assembly into microtubules upon introduction into cultured mammalian cells (1). However, there is no direct evidence concerning the contribution

* Corresponding author.

of the carboxy-terminal domains of mammalian α-tubulin isotypes to the proper functioning of microtubules in vivo.

Here we present the results of an investigation of the biological functions of the amino- and carboxy-terminal regions of mammalian tubulins. Using either site-directed mutagenesis or controlled exonuclease digestion, we introduced deletions in the amino-terminal or carboxy-terminal domains of tubulin polypeptides. These altered tubulins were introduced into mammalian cells by transfection and analyzed in transient assays and in stable cell lines for their ability to coassemble into microtubules. The data show that limited deletions introduced either at the amino terminus of α-tubulin or in the amino-terminal regulatory domain of β-tubulin result in their competent coassembly into microtubules with an efficiency only slightly less than that of wild-type tubulin polypeptides. In addition, these coassembly-competent altered polypepetides have a half-life in vivo that is indistinguishable from that of their wild-type counterparts.

MATERIALS AND METHODS

Generation of immunologically tagged carboxy-terminal deletions of the Mα4 tubulin isotype. To generate plasmids containing successive carboxy-terminal deletions of the Mα4 tubulin isotype, a plasmid containing a full-length cDNA encoding Mα4 (32) was linearized with *Sal*I (which cleaves at the 3′ boundary of the recombinant insert) and digested with exonuclease *Bal* 31. The reaction products were then subjected to partial digestion with *Eco*RI to yield a series of fragments containing the entire Mα4 coding region but truncated at the 3′ end. These fragments were cloned into bacteriophage M13mp8 and sequenced to establish the extent of 3′-end deletion and to identify those deletions suitable for fusion to sequences encoding immunoreactive tags (see below). Deleted molecules selected in this manner that lacked sequences encoding 2, 9, 12, 14, or 17 carboxy-terminal amino acids were digested with *Hin*dIII, blunt-ended by reaction with the Klenow fragment of DNA polymerase I, and partially digested with *Eco*RI for insertion into the Mβ1-pSV expression vector (17, 22). The latter construct, consisting of a full-length cDNA encoding Mβ1 flanked by simian virus 40 T-antigen promoter and terminator sequences was cleaved with *Eco*RV so as to remove the entire Mβ1 coding region with the exception of sequences encoding the carboxy-terminal 38 amino acids. The bulk of the Mβ1 coding sequence was then replaced by the carboxy-terminally deleted fragments encoding Mα4 so as to generate plasmids capable of expressing carboxy-terminally deleted variants of the Mα4 polypeptide tagged with the 38 carboxy-terminal amino acids of Mβ1.

To create equivalent constructs tagged by *Escherichia coli* Arg repressor sequences (as opposed to the Mβ1 carboxy-terminal sequence), a cleaved fragment containing an *E. coli* Arg repressor cDNA (5) was cleaved with *Hin*dIII, blunt-ended by treatment with the Klenow fragment of DNA polymerase I, and digested with *Kpn*I. This fragment was ligated with a fragment derived from Mβ1-pSV (17) obtained by digestion with *Eco*RV and *Kpn*I. This resulted in a plasmid in which the carboxy-terminal 38 amino acids of Mβ1 were replaced by a fragment encoding 78 amino acids of the *E. coli* Arg repressor. This hybrid plasmid was digested with *Spe*I, incubated with Klenow fragment, and partially cleaved with *Eco*RI, resulting in removal of the entire Mβ1 sequence, but retaining sequences encoding the *E. coli* Arg repressor. cDNA sequences encoding the Mα4 tubulin sequence with successive carboxy-terminal deletions (generated as described above) were inserted into this construct so as to produce plasmids capable of expressing Mα4 polypeptides carrying carboxy-terminal deletions fused to a 78-amino-acid segment of the *E. coli* Arg repressor.

Generation of expression plasmids encoding tubulins altered by the deletion of amino-terminal amino acids. (i) Amino-terminal deletions of Mβ1. Amino-terminal deletions of sequences encoding the Mβ1 tubulin isotype were obtained by site-directed mutagenesis (15). Briefly, a *Hin*dIII-*Bal*I fragment containing sequences encoding the wild-type amino terminus of Mβ1 (nucleotides [nt] −17 to +30) was isolated and cloned into bacteriophage M13mp19 containing a modified polylinker that includes a *Bal*I restriction site. Oligonucleotide-directed changes were introduced so as to delete nucleotides encoding amino acid 2, amino acids 2 and 3, amino acids 2 to 4, or amino acids 2 to 5. Subcloned fragments containing these deletions were substituted for wild-type Mβ1 amino-terminal sequences contained in the Mβ1-pSV expression plasmid. The correct substitution of fragments containing each deleted amino-terminal segment was confirmed by sequence analysis of the final construct.

(ii) Amino-terminal deletions of Mα4. Amino-terminal deletions of the Mα4 α-tubulin isotype were generated by site-directed mutagenesis (15) with a subcloned *Sac*I-*Hin*dIII fragment which encodes wild-type amino-terminal sequences of Mα4 (nt −20 to +66). Fragments containing deletions of nucleotides encoding amino acid 2, amino acids 2 and 3, or amino acids 2 to 4 of Mα4 were isolated and inserted into the expression plasmid Mα4-pSV (10) so as to replace the wild-type Mα4 amino-terminal sequence.

Transfection and immunofluorescence. Adherent HeLa cells were grown on glass cover slips in petri dishes in Dulbecco modified Eagle medium (DMEM) containing 10% defined calf serum. Cells were transfected by the calcium phosphate precipitation method (34). For transient transfection experiments, cells were fixed 48 h after transfection, permeabilized, and incubated with affinity-purified rabbit antiserum specific for Mβ1 (17), Mα4 (10), or the *E. coli* Arg repressor (5) together with a guinea pig general anti-α- or anti-β-tubulin antibody as described previously (10, 17). For the generation of stable cell lines, cells were cotransfected with plasmids engineered for the expression of altered α- and β-tubulin (see above) together with a selectable neomycin resistance marker at a ratio of 30:1. G418-resistant cell lines expressing altered α- or β-tubulin polypeptides were identified by indirect immunofluorescence with isotype-specific sera (10, 17).

Determination of the half-life of wild-type and altered α- and β-tubulin polypeptides in vivo. Duplicate dishes of adherent HeLa cells grown in DMEM were transfected (34) with 10 μg of various α- and β-tubulin constructs designed to express either wild-type or altered tubulin polypeptides. At 48 h after transfection, the cells were labeled for 5 h by incubation in methionine-free medium containing 125 μCi of [^{35}S]methionine (specific activity, 1.3 kCi/mmol) per ml and then allowed to grow for a further period in unlabeled DMEM. At 0, 12, 24, 36, and 48 h after the end of the labeling period, fractions containing polymerized and unpolymerized tubulin were prepared as described previously (17, 30). Labeled polypeptides expressed from transfected sequences were immunoprecipitated from these fractions by reaction with antisera specific for either Mβ1 or the *E. coli* Arg repressor protein. Immunoprecipitated material recovered by reaction with protein A-agarose beads was analyzed by resolution on 8% stacking sodium dodecyl sulfate (SDS)

gels (16). Signals corresponding to specifically immunoprecipitated tubulin polypeptides were quantitated by densitometry.

RESULTS

Assembly properties of altered β-tubulin polypeptides containing amino-terminal deletions. To construct amino-terminal deletions that would yield a correctly initiated β-tubulin polypeptide, we removed nucleotides encoding one or more amino acids directly 3′ to the initiator methionine residue. We selected the hematopoietic β-tubulin isotype Mβ1 (33) for these experiments for two reasons. First, we had in hand a specific antibody that uniquely detects this isotype (17), and second, the Mβ1 isotype is not detectably expressed in HeLa cells, although it assembles normally into microtubules when introduced by transfection (17). The products of the deletion reactions were in all cases confirmed by DNA sequencing. The amino-terminally deleted Mβ1-encoding sequences were cloned into the pSV expression vector (see Materials and Methods) and introduced into HeLa cells in a transient transfection assay. The ability of the deleted tubulins to coassemble was then tested by indirect double-label immunofluorescence. Mβ1 tubulins in which sequences encoding either amino acid 2 or amino acids 2 and 3 were deleted coassembled into interphase microtubules in a manner indistinguishable from that revealed by a general tubulin antibody (Fig. 1, compare panels a, c, and e with panels b, d, and f). Spindle microtubules of dividing cells expressing the transfected polypeptides were also coincidentally labeled (data not shown). To see whether the coassembly of altered polypeptides affected cell viability, we generated stable cell lines expressing these altered polypeptides. Examination of these cell lines by indirect immunofluorescence showed the uniform expression and normal coassembly of the altered β-tubulin polypeptides, while there was no evidence for any impairment of growth or viability, as judged by their doubling time. In contrast, when an Mβ1 tubulin carrying deletions encoding amino acids 2, 3, and 4 was transfected, two phenotypes were observed (Fig. 2). In some transfected cells, the deleted isotype assembled into microtubules with poor efficiency, as evidenced by weak staining with the Mβ1-specific antibody (Fig. 2, compare panels a and b). In other cells, the same polypeptide completely failed to assemble into microtubules and instead was located in strongly staining perinuclear foci that did not stain with the general tubulin antibody (Fig. 2, panels c and d). Therefore, removal of the Arg and Glu residues from the amino terminus of β-tubulin does not abolish the ability of the β-tubulin polypeptide to coassemble into microtubules, whereas further deletion severely compromises the ability to coassemble. More extensive amino-terminal deletions also resulted in polypeptides that completely failed to coassemble (data not shown).

To assess the relative efficiency with which altered coassembly-competent β-tubulin polypeptides were incorporated into polymerized microtubules, either transiently transfected cells or stable cell lines expressing amino-terminally deleted β-tubulins were labeled for 5 h with [^{35}S]methionine, and fractions containing unpolymerized and polymerized tubulin were prepared (30). Altered Mβ1 tubulin polypeptides were immunoprecipitated from these fractions with an Mβ1-specific antibody and quantitated by densitometry following SDS-polyacrylamide gel electrophoresis. Each transient transfection experiment was performed in triplicate so as to maximize quantitative accuracy (Fig. 3A to D). The data from these transient assays showed that the relative ratio of unpolymerized to polymerized Mβ1 tubulin lacking either amino acid 2 or amino acids 2 and 3 was in the range of 45:55 to 55:45, compared with a corresponding relative ratio of about 35:65 in cells expressing wild-type Mβ1 tubulin (Table 1). Data obtained in experiments with stable cell lines expressing the same truncated polypeptides were in reasonable agreement with the results of transient transfection experiments (Fig. 3E and F and Table 1). Thus, these amino-terminally deleted Mβ1 tubulin polypeptides are coassembled into microtubules somewhat less efficiently than their wild-type counterparts. In contrast, the level of Mβ1 lacking amino acids 2 to 4 was very low in the soluble tubulin pool and virtually undetectable in polymerized microtubules (Fig. 3D).

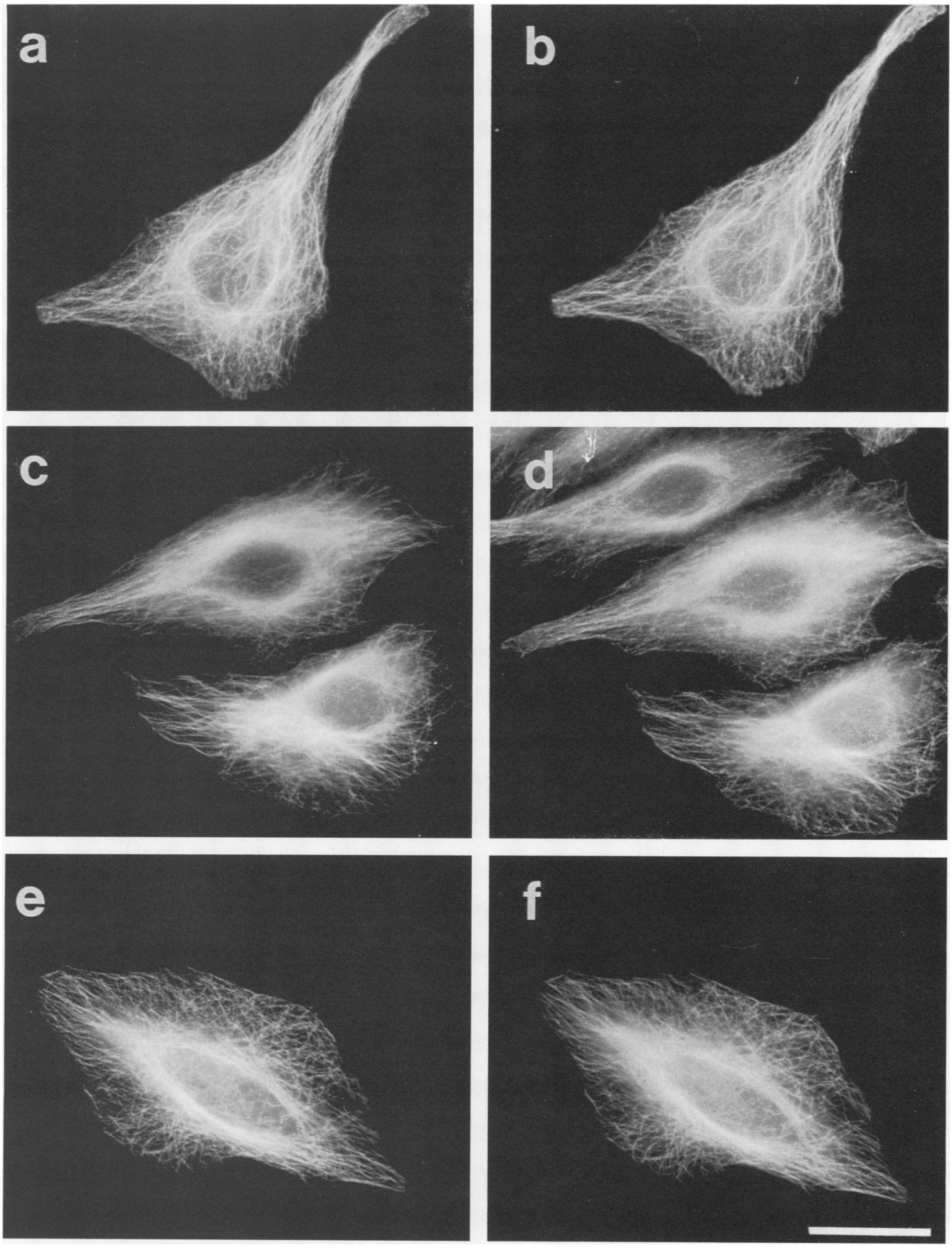
TABLE 1. Ability of α- and β-tubulin polypeptides carrying various amino-terminal deletions to coassemble into microtubules in HeLa cells

Isotype	Deleted residue(s)[a]	Assembly	Ratio, unpolymerized/polymerized[b]
Mβ1	None	+	35:65 (40:60)
	Arg (aa 2)	+	55:45 (50:50)
	Arg, Glu (aa 2 + 3)	+	45:55 (50:50)
	Arg, Glu, Ile (aa 2–4)	±	—[c]
	Arg, Glu, Ile, Val (aa 2–5)	−	N.D.[d]
Mα4	None	+	(40:60)
	Arg (aa 2)	+	(50:50)
	Arg, Glu (aa 2 + 3)	±	N.D.

[a] aa, Amino acid.
[b] Data are from transient assays (Fig. 3A to D) or from permanent cell lines (shown in parentheses) (Fig. 3E and F).
[c] —, Polymerized material present at an undetectably low level (Fig. 3D, tracks 4 to 6).
[d] N.D. Not determined.

Assembly properties of altered α-tubulin polypeptides containing amino-terminal deletions. We used a strategy similar to that described above to test the assembly properties of α-tubulins carrying deletions encoding amino acid 2 and amino acids 2 and 3. For these experiments, the deletions were introduced into the Mα4 isotype (32) since, as in the case of Mβ1, we previously described a specific antibody for this isotype, and its level of expression in cells in culture is relatively low (10).

FIG. 1. Coassembly into interphase microtubules of altered Mβ1 tubulin polypeptides lacking amino acid 2 or amino acids 2 and 3. Constructs carrying deletions of nucleotides encoding amino acid 2 or amino acids 2 and 3 of the Mβ1 tubulin isotype (33) were introduced into the pSV expression vector (22) and used in transient transfection assays of HeLa cells. At 48 h after transfection, cells were fixed and analyzed for the ability of the introduced isotype to coassemble into microtubules by indirect double-label immunofluorescence (24) with an antibody specific for Mβ1 together with a general tubulin antibody that does not discriminate among α-tubulin isotypes (17). (a, c, e) Anti-Mβ1 antibody; (b, d, f) identical to a, c, and e, respectively, but detected with a general anti-α-tubulin antibody. (a and b) Coassembly into interphase microtubules of wild-type Mβ1. (c and d) Coassembly into interphase microtubules of an altered Mβ1 polypeptide lacking amino acid 2. (e and f) Coassembly into microtubules of an altered Mβ1 polypeptide lacking amino acids 2 and 3. In all cases, the microtubules detected by the Mβ1 antibody and the general anti-α-tubulin antibody are coincident. Note the presence of nontransfected cells (compare panels c and d). Bar, 20 μm.


a
b
c
d
e
f

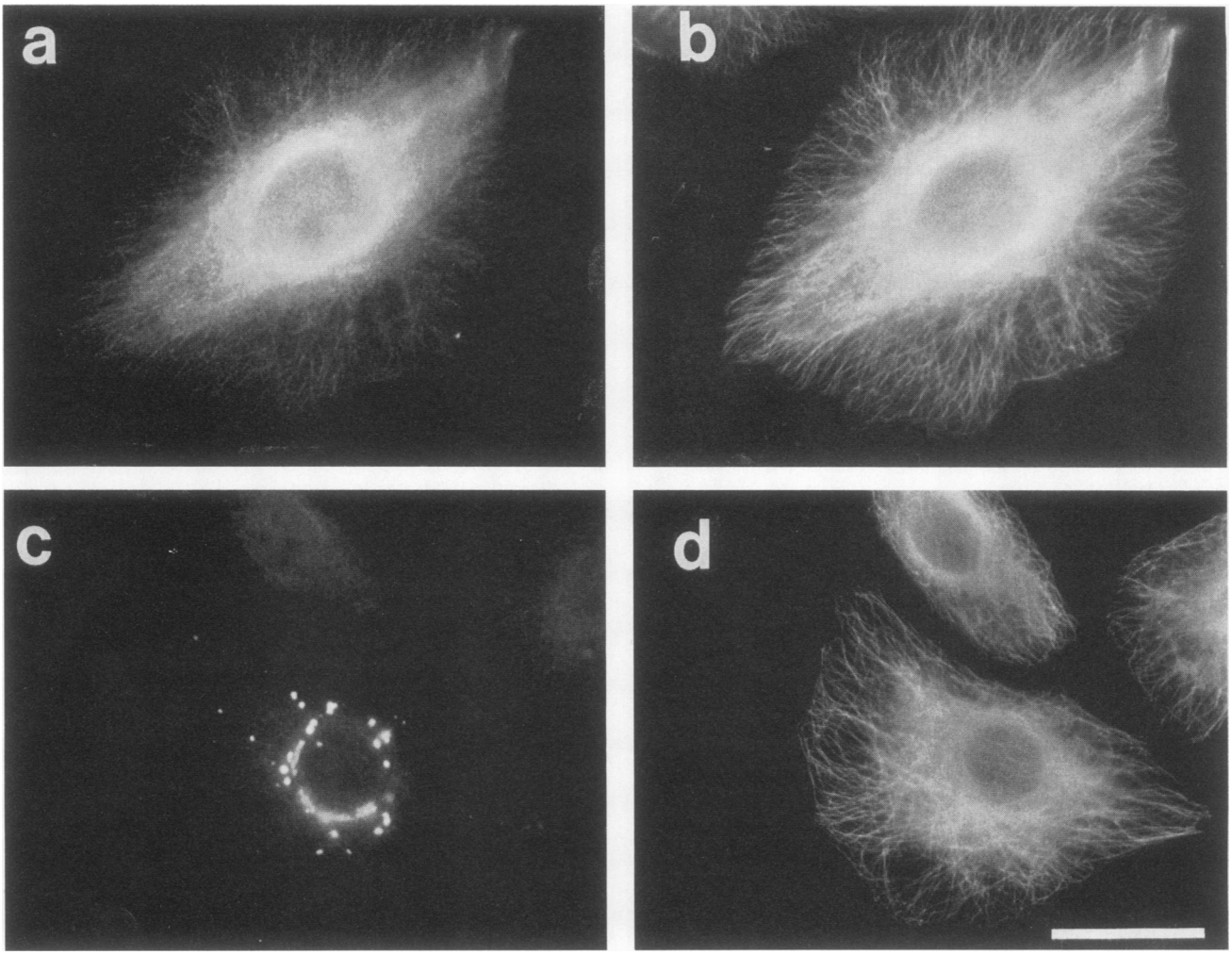


FIG. 2. Coassembly into interphase microtubules of altered Mβ1 β-tubulin polypeptides lacking amino acids 2, 3, and 4. A deleted construct lacking nucleotides encoding amino acids 2 to 4 (inclusive) of the Mβ1 isotype was cloned into the pSV expression vector (see Materials and Methods) and introduced into HeLa cells by transfection (34). The transfected cells were analyzed by indirect double-label immunofluorescence as described in the legend to Fig. 1. Fields representative of two frequently occurring phenotypes are shown. (a and c) Anti-Mβ1 antibody; (b and d) general anti-α-tubulin antibody. Panels a and c show fields identical to panels b and d, respectively. Bar, 20 μm.

The results of transient transfection experiments are shown in Fig. 4. Removal of the Arg residue from the amino terminus of Mα4 did not alter the ability of the polypeptide to coassemble into normal microtubules (Fig. 4c and d). To determine whether the expression of this altered α-tubulin affected cell growth or viability, we generated a stable cell line expressing this polypeptide. As in the case of transiently transfected cells, the stable cell line showed incorporation of the amino-terminally deleted α-tubulin into interphase and spindle microtubules, and there was no detectable influence on cell morphology or growth characteristics. The relative distribution of the coassembly-competent altered α-tubulin peptide lacking amino acid 2 between unassembled and polymerized tubulin pools was measured in this cell line in experiments analogous to those described above for amino-terminally deleted β-tubulin polypeptides. The data (Table 1) showed that the distribution of the altered Mα4 tubulin between the unpolymerized and polymerized fractions was approximately in the ratio 50:50, in contrast to a corresponding ratio of 40:60 for the wild-type Mα4 tubulin counterpart.

When nucleotides encoding amino acids 2 (Arg) and 3 (Glu) were deleted from Mα4, expression of the altered constructs in HeLa cells resulted in two phenotypes similar to those observed in the case of the Mβ1 isotype lacking amino acids 2 to 4 (Fig. 2). In some cells, there was weak but detectable coassembly into normal microtubules (data not shown); in others, however, there was a complete failure of the altered α-tubulin to coassemble, with the mutant polypeptide appearing in dense foci located mostly around the cell nucleus (Fig. 4e).

Assembly properties of α-tubulin polypeptides containing carboxy-terminal deletions. A major difficulty in studying the effect of carboxy-terminal deletions on the assembly of tubulin isotypes in higher eucaryotic cells in vivo is that the epitopes recognized by isotype-specific sera reside in the carboxy-terminal region (4; W. Gu, unpublished observations). Hence, the removal of this region results in loss of the ability to specifically detect the deleted isotype. To overcome this difficulty, a small region encoding the carboxy-terminus of Mβ1 that includes the epitope(s) recognized by the Mβ1-specific antibody was used as an immunological tag by coupling it to molecules of the Mα4 α-tubulin isotype carrying successive deletions at the carboxy terminus (Fig. 5A). The ability of the encoded polypeptides to coassemble

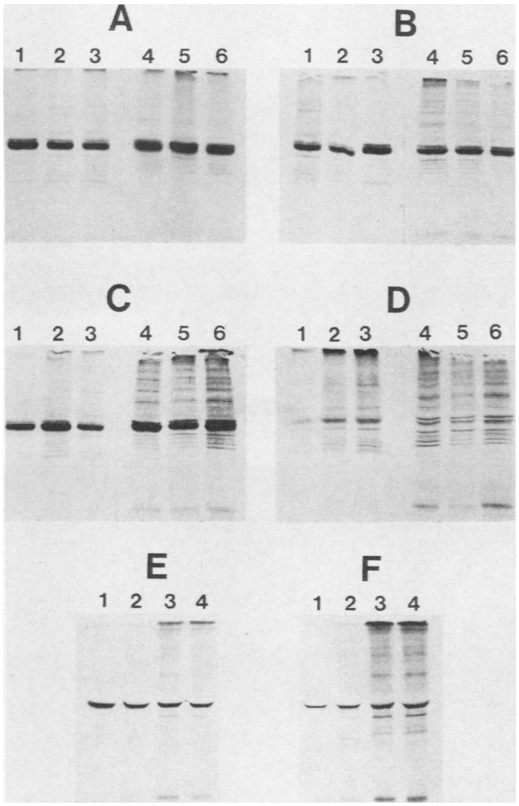


FIG. 3. Quantitative analysis of wild-type and altered Mβ1 tubulin polypeptides in the unpolymerized and polymerized tubulin fractions of transfected HeLa cells. (A to D) Constructs engineered for the expression of Mβ1 polypeptides lacking either amino acid 2, amino acids 2 and 3, or amino acids 2 to 4 were introduced into HeLa cells by transfection (34). At 48 h after transfection, the cells were labeled with [^{35}S]methionine for 5 h. Fractions containing the unpolymerized and polymerized tubulin were prepared (30), and the Mβ1 polypeptides were isolated by reaction with Mβ1-specific antibody (17). For quantitative accuracy, each transfection was performed in triplicate. Panel A, wild-type Mβ1; panel B, Mβ1 lacking amino acid 2; panel C, Mβ1 lacking amino acids 2 and 3; panel D, Mβ1 lacking amino acids 2 to 4. Tracks 1 to 3, Unpolymerized fractions; tracks 4 to 6, polymerized fractions. (E and F) Stable cell lines were constructed expressing Mβ1 lacking amino acid 2 (E) and amino acids 2 and 3 (F) (see Materials and Methods). Duplicate dishes of cells were labeled and fractionated, and the unpolymerized and polymerized Mβ1 polypeptides were analyzed following immunoprecipitation with the specific anti-Mβ1 antibody. Tracks 1 and 3, Unpolymerized fractions; tracks 2 and 4, polymerized fractions.

into microtubules was then assayed in transient transfection assays with the Mβ1-specific antibody. The data show that the tagged Mα4 isotype lacking 12 amino acids at the carboxy terminus coassembled in a manner indistinguishable from the staining pattern obtained with a general tubulin antibody (Fig. 6a and b); the same result was obtained with analogous constructs lacking 2 or 9 carboxy-terminal amino acids (data not shown). In contrast, deletion of 14 amino acids from the carboxy terminus of the Mα4 isotype resulted in inefficient coassembly of the transfected polypeptide into microtubules (Fig. 6c and d). More extensive deletions (i.e., removal of 17 amino acids from the carboxy terminus of Mα4) resulted in complete failure to coassemble into microtubules.

The above deletion experiments depended on the fusion of an immunological tag derived from the carboxy terminus of the Mβ1 isotype to the truncated carboxy terminus of Mα4. Because this tag derived from another tubulin polypeptide and is rich in acidic residues (a property common to the carboxy-terminal regions of all tubulin polypeptides), we were concerned that data obtained from the above transfection experiments might be influenced by the addition of the Mβ1 tag. Therefore, we made constructs in which an alternative immunological tag, completely unrelated to any tubulin molecule, was used in place of the Mβ1 sequence. This tag derives from the Arg repressor of *E. coli* (5) (Fig. 5B). When a construct consisting of Mα4 lacking 14 carboxy-terminal amino acids and tagged with the *E. coli* Arg repressor epitope was transfected into HeLa cells, the result was identical to that depicted in Fig. 6c; however, deletion of 12 or fewer carboxy-terminal amino acids resulted in normal coassembly into microtubules (Fig. 6e and f). We conclude that up to 12 carboxy-terminal amino acids can be removed from Mα4 without disrupting the ability to coassemble and that the coupling of a substantial number of additional amino acid residues, from whatever source, to the carboxy terminus of Mα4 has no detectable effect on the assembly properties of the molecule.

Stability in vivo of altered α- and β-tubulin polypeptides. The above experiments show that certain altered α- and β-tubulins are competent for coassembly into interphase and mitotic spindle microtubules. However, the possibility exists that such altered tubulins are in some way functionally compromised. To examine the stability of these altered α- and β-tubulins in vivo, we performed pulse-chase experiments and compared their half-lives with those of their wild-type counterparts. The data from these experiments are shown in Fig. 7. Removal of amino acids 2 (Arg) and 3 (Glu) from the amino terminus of the Mβ1 isotype had no significant effect on its stability in vivo relative to wild-type Mβ1 (Fig. 7A and B). Quantitation by densitometry of the autoradiographs showed that the half-life in each case was of the order of 27 h.

Direct measurement of the stability of transfected Mα4 tubulin polypeptides is complicated by the low level of endogenous wild-type Mα4 expression in HeLa cells (shown, for example, in nontransfected cells in Fig. 4a). Therefore, an immunological tag consisting of the 38 carboxy-terminal residues derived from Mβ1 was fused to either the wild-type Mα4 sequence or Mα4 lacking sequences encoding amino acid 2. In common with the experiment shown in Fig. 6a, the encoded chimeric polypeptides coassembled normally into microtubules in transient transfection experiments. No significant difference in stability was observed in pulse-chase experiments with either the tagged wild-type Mα4 polypeptide or the altered Mα4 polypeptide lacking amino acid 2 (Fig. 7C and D); once again, densitometric quantitation of the α-tubulin-specific bands on the autoradiograph showed half-lives of about 27 h. Finally, a similar half-life was observed for an altered Mα4 α-tubulin lacking 12 amino acids from the carboxy terminus, immunologically tagged with carboxy-terminal sequences derived from Mβ1 (Fig. 7E). Half-life measurements were unobtainable for altered tubulins which completely failed to assemble because of their low abundance. Therefore, we cannot say whether the failure of these altered tubulins to assemble led to their instability, or whether their instability may have led to our failure to observe them in microtubules.

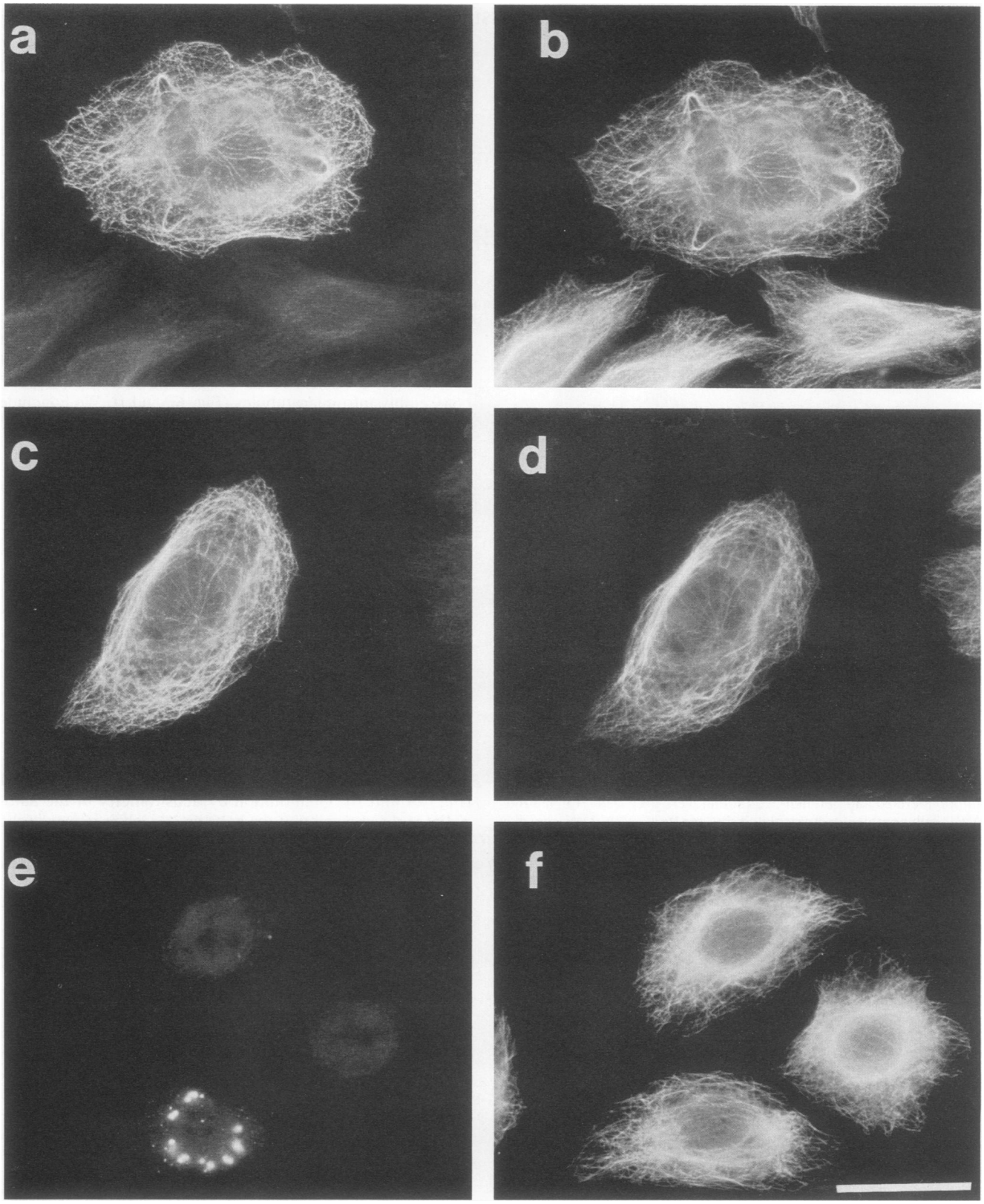


FIG. 4. Assembly into interphase microtubules of altered Mα4 polypeptides lacking amino acid 2 or amino acids 2 and 3. Constructs lacking nucleotides encoding amino acid 2 or amino acids 2 and 3 of the Mα4 tubulin isotype were cloned into the pSV expression vector and introduced into HeLa cells by transfection (34). Transfected cells were analyzed by indirect double-label immunofluorescence with the Mα4-specific antibody and a general anti-β-tubulin antibody that does not discriminate among β-tubulin isotypes (10). (a, c, e) Anti-Mα4-specific antibody; (b, d and f) same fields as a, c, and e, respectively, detected with the general β-tubulin antibody. Panels a and b, Coassembly into interphase microbutules of wild-type Mα4; panels c and d, coassembly into interphase microtubules of an altered Mα4 polypeptide lacking amino acid 2 (Arg); panels e and f, failure to coassemble, with perinuclear aggregates resulting from expression of an altered Mα4 polypeptide lacking amino acids 2 (Arg) and 3 (Glu). Note the low level of Mα4 expression in nontransfected cells in panel a. Bar, 20 μm.

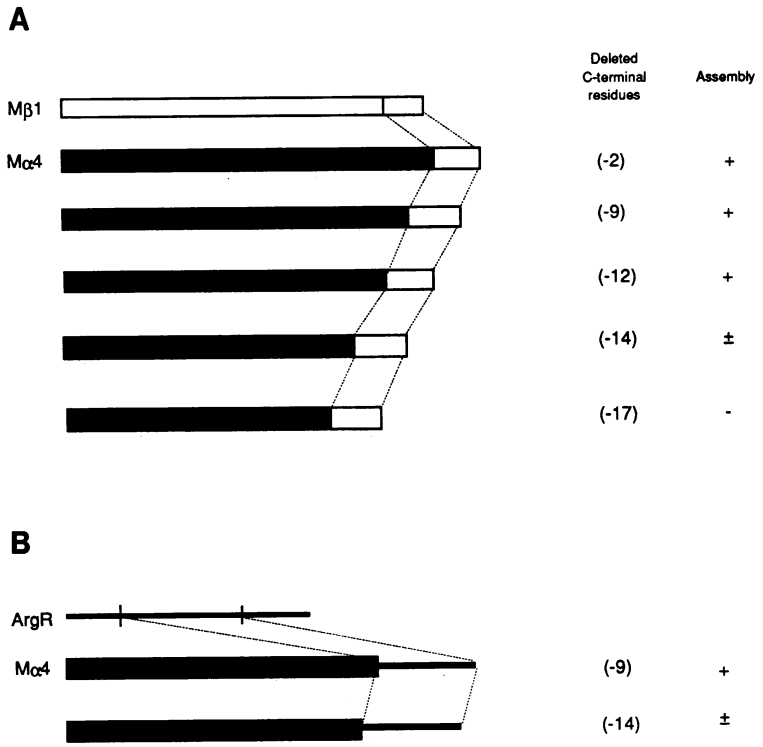


FIG. 5. Constructs used for the analysis of carboxy-terminal deletion mutants of Mα4. (A) The wild-type Mβ1 polypeptide is shown at the top (open box). Mα4 polypeptides (not drawn to scale), each containing successively greater carboxy-terminal deletions and each fused to the carboxy-terminal portion of Mβ1 that contains the anti-Mβ1 epitope(s), are shown below (cross-hatched). The number of deleted Mα4 carboxy-terminal residues is shown at the right, together with the result of transfection experiments to assay their ability to coassemble into microtubules (see Fig. 6). (B) The *E. coli* Arg repressor polypeptide (5) is shown (thick line, top). Below, sequences encoding a segment of this polypeptide were fused to sequences encoding the Mα4 polypeptide (cross-hatched) carrying deletions encoding either 9 or 14 carboxy-terminal amino acids.

DISCUSSION

Recent evidence suggests that the level of unpolymerized β-tubulin in eucaryotic cells is subject to autoregulation via a mechanism in which the nascent β-tubulin polypeptide itself acts as a signal to regulate the stability of its own mRNA (8, 26, 35, 36). Though there is evidence that the level of tubulin subunits is inversely related to the stability of tubulin mRNA in certain lower eucaryotes (e.g., in sea urchin embryos [9]), the physiological or developmental consequences of abolishing the autoregulatory phenomenon are unknown. The domain responsible for β-tubulin autoregulation has been delineated by deletion experiments that show the first four amino acids of the β-tubulin polypeptide to be both necessary and sufficient to influence the level of β-tubulin mRNA (35, 36). Disruption of this domain leads to failure of the normal autoregulatory phenomenon. The experiments reported here show that expression of a β-tubulin polypeptide in which sequences encoding either amino acid 2 or amino acids 2 and 3 have been deleted results in coassembly into interphase and spindle microtubules with only slightly reduced efficiency relative either to their unaltered counterpart (summarized in Table 1) or to endogenous tubulin isotypes (17). In addition, these deletions have no detectable effect either on the stability of the tubulin polypeptide in vivo (Fig. 7) or on the growth or morphology of stable cell lines expressing these polypeptides.

The level of foreign β-tubulin polypeptides expressed as a consequence of transfection is highly variable but probably never represents the bulk of β-tubulin present in the cell. Therefore, the microtubules in all our experiments are mixed copolymers of altered and wild-type β-tubulin polypeptides, and it remains possible that amino-terminally deleted molecules are functionally deficient in a way that would only become clear in a situation where their expression became dominant over their wild-type counterparts. Experiments are under way to test this possibility. Alternatively, the amino-terminal domain of β-tubulin could be functionally required in specialized microtubules that are not found in cultured cells. Nonetheless, it seems likely that amino acids 2 and 3 of β-tubulin serve mainly in a regulatory role, and our data suggest that the evolutionary conservation of the amino-terminal domain of β-tubulin is at least in part a consequence of selective pressure based on its role in autoregulation, rather than an absolute requirement for assembly.

Because the initiator methionine residue is required for the initiation of protein synthesis, we were unable to test the assembly properties of α- or β-tubulin polypeptides lacking the authentic amino-terminal amino acid. The initiator methionine residue is removed from many proteins by an enzyme (methionine aminopeptidase) with defined substrate preferences (6); at amino acid position 2, Lys, Arg, and Leu prevent cleavage of the amino-terminal methionine, while Ala, Gly, Pro, Ser, Thr, and Val promote its removal. When other amino acids occur at position 2, cleavage of the initiator methionine is less predictable. In all naturally occurring α- and β-tubulin polypeptides, Arg is found next to the initiator methionine (Lewis and Cowan, in press); consequently, all wild-type tubulin polypeptides begin with a

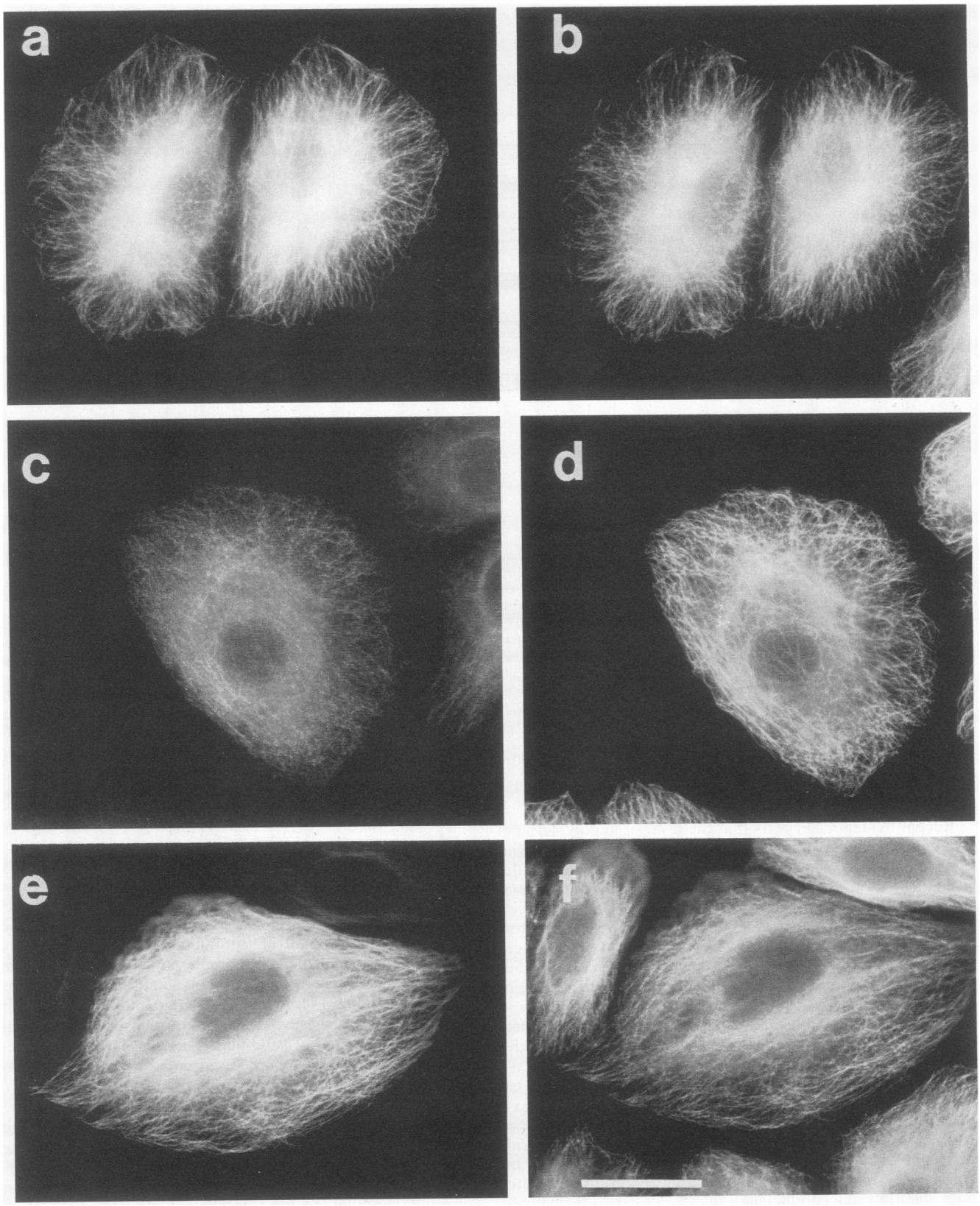


FIG. 6. Assembly of altered Mα4 tubulin polypeptides containing deleted carboxy-terminal amino acids. Immunologically tagged constructs containing carboxy-terminal deletions of the Mα4 isotype (Fig. 5) were introduced into HeLa cells in transient transfection experiments (34), and the ability of the transfected polypeptides to assemble into microtubules was assayed by indirect double-label immunofluorescence (24) with either the Mβ1-specific antibody (17) (a and c) or the anti-*E. coli* Arg repressor antibody (5) (e). (b, d and f) Fields identical to a, c, and e, respectively, detected with a general anti-α-tubulin antibody that does not discriminate among α-tubulin isotypes (17). Panels a and c, Transfected cells expressing the Mα4 polypeptide lacking 9 or 14 carboxy-terminal amino acids, respectively. The deleted Mα4 polypeptides were in both cases immunologically tagged with the Mβ1 carboxy-terminal region (Fig. 5A). Panel e shows expression of the Mα4 polypeptide lacking 9 carboxy-terminal amino acids, immunologically tagged with a region of the *E. coli* Arg repressor (Fig. 5B). Bar, 20 μm.

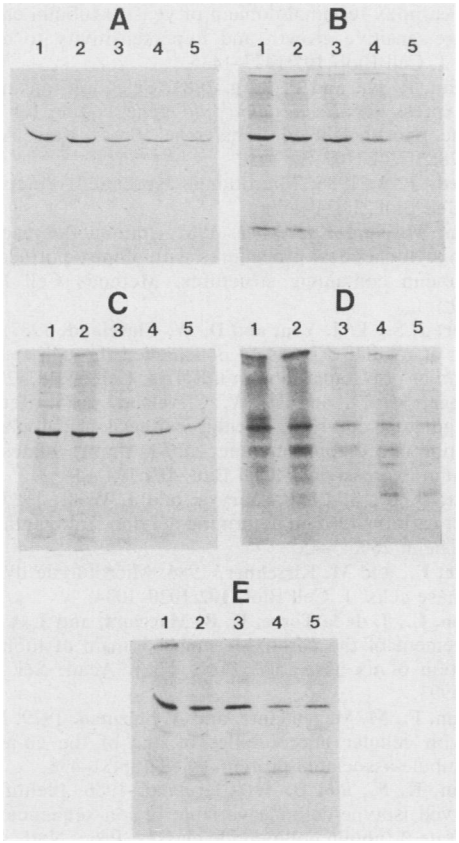


FIG. 7. Stability in vivo of wild-type and altered tubulin polypeptides. HeLa cells transfected with constructs containing either wild-type or altered sequences encoding either the Mβ1 isotype or the Mα4 isotype immunologically tagged with the carboxy-terminal domain of Mβ1 (see text) were incubated for 5 h in the presence of [^{35}S]methionine. At the end of this period, the labeled medium was removed and replaced with normal growth medium. At various times following the end of the labeling period (0, 12, 24, 36, and 48 h, shown in tracks 1 to 5, respectively), cells were harvested and extracts were prepared containing the soluble (unpolymerized) tubulin (see Materials and Methods). Tubulin polypeptides expressed via the introduction of transfected sequences were immunoprecipitated from these extracts by reaction with the Mβ1-specific antibody (7). The immunoprecipitated products were resolved on stacking 8% SDS-polyacrylamide gels (16), which were dried and prepared for fluorography. (A) Mβ1 (wild type). (B) Mβ1 lacking amino acids 2 (Arg) and 3 (Glu). (C) Mα4 (wild type). (D) Mα4 lacking amino acid 2 (Arg). (E) Mα4 lacking 12 carboxy-terminal amino acids.

methionine residue. When polypeptides are altered so that amino acid 2, amino acids 2 and 3, or amino acids 2, 3, and 4 have been deleted, the initiator methionine is followed by Glu, Ile, and Val, respectively. In the former two cases (neither of which intereferes with efficient coassembly into microtubules; Table 1), the fate of the initiator methionine residue is uncertain, while in the latter case the methionine residue is most probably cleaved off. Therefore, the failure of the altered β-tubulin polypeptide lacking amino acids 2, 3, and 4 to coassemble efficiently into microtubules (Fig. 2) could be a consequence of the posttranslational removal of the initiator methionine residue.

Comparison of the amino-terminal sequences of α-tubulins with those of β-tubulins shows that three of the first four amino acids (Met, Arg, and Glu) are identical, whereas the fourth differs (β-tubulin, Ile; α-tubulin, Cys, Ile, Val, or Ala). This is true for all tubulin isotypes from all species studied (Lewis and Cowan, in press). The level of α-tubulin mRNA, like that of β-tubulin mRNA, is autoregulated by the abundance of unpolymerized tubulin (26). However, the contribution of the amino terminus of α-tubulin to regulating the level of its mRNA has not been studied. Our results show that, as in the case of β-tubulin, the removal of amino acid 2 (Arg) only slightly reduces the ability of the α-tubulin polypeptide to coassemble into microtubules; however, removal of amino acids 2 (Arg) and 3 (Glu) has a drastic effect on assembly (Table 1). Therefore, the amino-terminal region of α-tubulin would seem to have a more direct structural role than the corresponding domain of β-tubulin.

There is evidence from in vitro experiments that the highly acidic carboxy-terminal domains of α- and β-tubulin also play a role in regulating tubulin assembly, probably via an ionic interaction with MAPs (18–20). However, our results demonstrate that removal of the 12 carboxy-terminal amino acids from the Mα4 α-tubulin isotype does not affect its ability to coassemble into interphase or mitotic microtubules of HeLa cells (Fig. 6). In our experiments, the deletions resulted in the net removal of 7 acidic residues from Mα4. The fusion of an immunological tag consisting of the carboxy-terminal region of Mβ1 to the carboxy-terminally deleted Mα4 tubulin polypeptide results in the addition of a further 13 acidic residues to the chimeric polypeptide, in effect overcompensating for the loss of negative charge incurred by deletion. On the other hand, substitution of a region of the *E. coli* Arg repressor as an alternative immunological tag unrelated to any tubulin sequence results in the addition of three basic charges. Therefore, the net charge of the carboxy-terminal region is not a factor in determining the assembly competence of α-tubulin. Moreover, the addition of immunological tags from either Mβ1 or the *E. coli* Arg repressor polypeptide results in chimeric molecules that are up to 66 amino acids longer than the wild-type tubulin molecules. The observation that these greatly extended polypeptides are competent for coassembly into microtubules reinforces the conclusion that the acidic carboxy termini of α-tubulins lie on the outside of the molecule (2). Finally, we note that alteration of the Mα4 polypeptide by the substitution of alternative carboxy-terminal sequences presumably blocks the entry of these altered polypeptides into the normal cycle of carboxy-terminal tyrosination and detyrosination that is characteristic of most α-tubulins in vivo (3, 11, 14).

ACKNOWLEDGMENTS

We thank John Thomas for help with quantitative densitometry measurements, Joel Oppenheim for the *E. coli* Arg R clone and anti-*E. coli* Arg R antibody, and Diane Alexy for typing the manuscript.

This work was supported by grants from the National Institutes of Health and the American Heart Association.

LITERATURE CITED

1. **Bond, J. F., J. L. Fridovich-Keil, L. Pillus, R. C. Mulligan, and F. Solomon.** 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse microtubules in vivo. Cell **44**:461–468.
2. **Breitling, F., and M. Little.** 1986. Carboxyterminal regions on the surface of tubulin and microtubules: epitope locations of YOL1134, DMIA and DMIB. J. Mol. Biol. **189**:367–370.
3. **Bulinski, J. C., and G. G. Borisy.** 1987. Assembly and turnover of detyrosinated tubulin in vivo. J. Cell Biol. **105**:265–276.
4. **Burgoyne, R. D., M. A. Cambray-Deakin, S. A. Lewis, S. Sarkar, and N. J. Cowan.** 1988. Differential distribution of β-tubulin isotypes in cerebellum. EMBO J. **7**:2311–2319.

5. **Dongbin, L., J. D. Oppenheim, T. Eckhardt, and W. K. Mass.** 1987. Nucleotide sequence of the *argR* gene of *Escherichia coli* K-12 and isolation of its product, the arginine repressor. Proc. Natl. Acad. Sci. USA **84**:6697–6701.
6. **Flinta, C., B. Persson, H. Jornvall, and G. Von Heijne.** 1986. Sequence determinants of N-terminal protein processing. Eur. J. Biochem. **154**:193–196.
7. **Fridovich-Keil, J. L., F. K. Julian, F. Bond, and F. Solomon.** 1987. Domains of β-tubulin essential for conserved functions in vivo. Mol. Cell. Biol. **7**:3792–3798.
8. **Gay, D. A., T. J. Yen, J. T. Y. Lau, and D. W. Cleveland.** 1987. Sequences that confer β-tubulin autoregulation through modulated mRNA stability reside within exon 1 of a β-tubulin mRNA. Cell **50**:671–679.
9. **Gong, Z., and B. Brandhurst.** 1987. Stimulation of tubulin gene transcription by deciliation of sea urchin embryos. Mol. Cell. Biol. **7**:4238–4246.
10. **Gu, W., S. A. Lewis, and N. J. Cowan.** 1988. Generation of antisera that discriminate among mammalian α-tubulins: introduction of specialized isotypes into cultured cells results in their coassembly without disruption of normal microtubule function. J. Cell Biol. **106**:2011–2022.
11. **Gunderson, G. G., S. Khawaja, and J. C. Bulinski.** 1987. Postpolymerization detyrosination of α-tubulin: a mechanism for subcellular differentiation of microtubules. J. Cell Biol. **105**:251–264.
12. **Katz, W. S., and F. Solomon.** 1988. Diversity among β-tubulins: a carboxy-terminal domain of yeast β-tubulin is not essential in vivo. Mol. Cell. Biol. **8**:2730–2736.
13. **Kirschner, M., and T. Mitchison.** 1986. Beyond self-assembly: from microtubules to morphogenesis. Cell **45**:329–342.
14. **Kumar, N., and M. Flavin.** 1982. Modulation of some parameters of assembly of microtubules in vitro by tyrosination of tubulin. Eur. J. Biochem. **128**:215–222.
15. **Kunkel, A. T.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
16. **Laemmli, U. K.** 1970. Change of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Lewis, S. A., W. Gu, and N. J. Cowan.** 1987. Free intermingling of mammalian β-tubulin isotypes among functionally distinct microtubules. Cell **49**:539–548.
18. **Lewis, S. A., D. Wang, and N. J. Cowan.** 1988. Microtubule-associated protein MAP2 shares a microtubule-binding motif with tau protein. Science **242**:829–984.
19. **Littauer, U. Z., D. Giveon, M. Thierauf, I. Ginzburg, and H. Ponstingl.** 1986. Common and distinct tubulin binding sites for microtubule-associated proteins. Proc. Natl. Acad. Sci. USA **83**:7162–7166.
20. **Maccioni, R. B., C. I. Rivas, and J. C. Vera.** 1988. Differential interaction of synthetic peptides from the carboxy-terminal regulatory domain of tubulin with microtubule-associated proteins. EMBO J. **7**:1957–1963.
21. **Matsuzaki, F., S. Matsumoto, and I. Yahara.** 1988. Truncation of the carboxy-terminal domain of yeast β-tubulin causes temperature-sensitive growth and hypersensitivity to antimitotic drugs. J. Cell Biol. **107**:1427–1435.
22. **Mulligan, R. G., and P. Berg.** 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyl transferase. Proc. Natl. Acad. Sci. USA **78**:2072–2076.
23. **Olmsted, J. B.** 1986. Microtubule-associated proteins. Annu. Rev. Cell Biol. **2**:421–457.
24. **Osborn, M., and K. Weber.** 1982. Immunofluorescence and immunocytochemical procedures with affinity-purified antibodies: tubulin containing structures. Methods Cell Biol. **244**: 98–129.
25. **Pachter, J. S., T. J. Yen, and D. W. Cleveland.** 1987. Autoregulation of tubulin expression is achieved through specific degradation of polysomal tubulin mRNAs. Cell **51**:283–292.
26. **Pittenger, M. F., and D. W. Cleveland.** 1985. Retention of autoregulatory control of tubulin synthesis in cytoplasts: demonstration of a cytoplasmic mechanism that regulates the level of tubulin expression. J. Cell Biol. **101**:1941–1952.
27. **Sackett, D. L., B. Bhattacharyya, and J. Wolff.** 1985. Tubulin subunit carboxyltermini determine polymerization efficiency. J. Biol. Chem. **260**:43–45.
28. **Schulze, E., and M. Kirschner.** 1986. Microtubule dynamics in interphase cells. J. Cell Biol. **102**:1020–1034.
29. **Serrano, L., J. de la Torre, R. B. Maccioni, and J. Avila.** 1984. Involvement of the carboxyterminal domain of tubulin in the regulation of its assembly. Proc. Natl. Acad. Sci. USA **81**: 5989–5993.
30. **Solomon, F., M. Magendantz, and A. Salzman.** 1979. Identification with cellular microtubules of one of the co-assembling microtubule-associated proteins. Cell **18**:431–438.
31. **Sullivan, K. F., and D. W. Cleveland.** 1986. Identification of conserved isotype-defining variable region sequences for four vertebrate β-tubulin polypeptide classes. Proc. Natl. Acad. Sci. USA **83**:4327–4331.
32. **Villasante, A., D. Wang, P. Dobner, P. Dolph, S. A. Lewis, and N. J. Cowan.** 1986. Six mouse α-tubulin mRNAs encode five distinct tubulin isotypes: testis-specific expression of two sister genes. Mol. Cell. Biol. **6**:2409–2419.
33. **Wang, D., A. Villasante, S. A. Lewis, and N. J. Cowan.** 1986. The mammalian β-tubulin repertoire: hematopoietic expression of a novel, heterologous β-tubulin isotype. J. Cell. Biol. **103**: 1903–1910.
34. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from prokaryotes and eukaryotes. Cell **16**:777–785.
35. **Yen, T. J., D. A. Gay, J. S. Pachter, and D. W. Cleveland.** 1988. Autoregulated changes in stability of polyribosome-bound β-tubulin mRNAs are specified by the first 13 translated nucleotides. Mol. Cell. Biol. **8**:1224–1235.
36. **Yen, T. J., P. S. Machlin, and D. W. Cleveland.** 1988. Autoregulated instability of β-tubulin mRNAs by recognition of the nascent amino terminus of β-tubulin. Nature (London) **334**: 580–585.