MOLECULAR AND CELLULAR BIOLOGY, June 1994, p. 4076–4086
0270-7306/94/$04.00+0

Vol. 14, No. 6

An Amino-Terminal Tetrapeptide Specifies Cotranslational Degradation of β-Tubulin but Not α-Tubulin mRNAs

CINDY J. BACHURSKI,† NICHOLAS G. THEODORAKIS, RICHARD M. R. COULSON,‡ AND DON W. CLEVELAND*

Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 29 November 1993/Returned for modification 25 January 1994/Accepted 12 March 1994

The steady-state level of α- and β-tubulin synthesis is autoregulated by a posttranscriptional mechanism that selectively alters α- and β-tubulin mRNA levels in response to changes in the unassembled tubulin subunit concentration. For β-tubulin mRNAs, previous efforts have shown that this is the result of a selective mRNA degradation mechanism which involves cotranslational recognition of the nascent amino-terminal β-tubulin tetrapeptide as it emerges from the ribosome. Site-directed mutagenesis is now used to determine that the minimal sequence requirement for conferring the full range of β-tubulin autoregulation is the amino-terminal tetrapeptide MR(E/D)I. Although tubulin-dependent changes in α-tubulin mRNA levels are shown to result from changes in cytoplasmic mRNA stability, transfection of wild-type and mutated α-tubulin genes reveals that α- and β-tubulin mRNA degradation is not mediated through a common pathway. Not only does the amino-terminal α-tubulin tetrapeptide MREC fail to confer regulated mRNA degradation, neither wild-type α-tubulin transgenes nor an α-tubulin gene mutated to encode an amino-terminal MREI yields mRNAs that are autoregulated. Further, although slowing ribosome transit accelerates the autoregulated degradation of endogenous α- and β-tubulin mRNAs, degradation of α-tubulin transgene mRNAs is not enhanced, and in one case, the mRNA is actually stabilized. We conclude that, despite similarities, α- and β-tubulin mRNA destabilization pathways utilize divergent determinants to link RNA instability to tubulin subunit concentrations.

The two major microtubule subunits, α- and β-tubulin, are encoded in higher eukaryotes by multigene families whose expression is regulated at both the transcriptional and posttranscriptional levels (reviewed in references 3, 4, and 34). Although transcriptional activation determines the repertoire of tubulin isotypes expressed in each cell type (reviewed in reference 31), a posttranscriptional autoregulatory mechanism controls the rate of tubulin subunit synthesis relative to the unassembled tubulin subunit concentration. Elevation of tubulin subunit concentration by colchicine- or nocodazole-induced microtubule disassembly (1, 6) or by direct microinjection of unassembled tubulin into cells (7) causes a concomitant loss of both α- and β-tubulin mRNAs. Conversely, taxol, a drug which depletes the unassembled tubulin subunit pool by inducing assembly of microtubules, causes tubulin mRNA levels to rise (6).

Several lines of evidence suggest that the autoregulation of both α- and β-tubulin mRNA abundance is posttranscriptional. In nuclear run-on transcription assays using probes that cross-hybridized with all isotypes, no differences in the overall transcription rates of α- and β-tubulin genes were seen between nuclei isolated from colchicine-treated and untreated cells (5). Studies with enucleated cells confirmed the cytoplasmic location of the β-tubulin autoregulatory machinery: cytoplasts were able to respond to colchicine by decreasing the synthesis of β-tubulin (2, 26). However, it remains unproven whether changes in α-tubulin RNA levels result from nuclear or cytoplasmic events.

Autoregulated destabilization of β-tubulin mRNA is dependent on continued translation elongation, since the treatment of cells with drugs that either freeze mRNAs on polysomes (13) or disrupt mRNA binding to ribosomes prevents autoregulation (25). However, the 90% inhibition of protein synthesis caused by the treatment of cells with a low concentration of cycloheximide, which slows ribosome translocation but leaves polysomes intact, actually enhances autoregulated instability (13). The simplest view is that only β-tubulin mRNAs attached to elongating ribosomes are substrates for regulated degradation. As with β-tubulin mRNA, autoregulation of α-tubulin mRNA is blocked by complete inhibition of translation (15, 25).

The mechanism of β-tubulin autoregulation has been studied in detail by transient DNA transfection. The domain that specifies autoregulation of β-tubulin mRNA is contained within the first 13 translated nucleotides encoding the first four amino acids, Met-Arg-Glu-Ile (MREI) (36). Site-directed mutagenesis to introduce point mutations into this autoregulatory domain has revealed that the recognition element is actually the nascent peptide, since all mutations that preserve the arginine-coding potential of the second codon retain autoregulated instability; similarly, third-codon mutations that maintain the encoded glutamate (or a closely related aspartate) also remain substrates for autoregulation (37). Recently, direct evidence that the binding of cellular factor(s) to the nascent peptide is the event triggering degradation was obtained by microinjection of a monoclonal antibody that binds to the amino-terminal β-tubulin (but not nascent α-tubulin) peptide. Antibody binding to the nascent peptide specifically prevented tubulin-dependent degradation of β-tubulin mRNAs, while autoregulation of α-tubulin mRNAs continued undisrupted (33).

* Corresponding author. Mailing address: Department of Biological Chemistry, Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Phone: (410) 955-7554. Fax: (410) 955-1090.

† Present address: Division of Pulmonary Biology, Children's Hospital Research Foundation, Cincinnati, OH 45229.

‡ Present address: Department of Pathology, Cambridge University, Cambridge CB1 1QP, England.

All α-tubulins encode an amino-terminal MRE, but most diverge from β-tubulin at the fourth codon (I for known β-tubulins, but C for α-tubulin in vertebrates and either C, V, or I for α-tubulin in lower eukaryotes). No experiments to date have addressed the possibility that the minimal autoregulatory domain is actually MRE or MREX (where X is either I, C, or V), thereby directing recognition and degradation of α-tubulin mRNAs by the same cellular factor(s) as β-tubulin mRNAs. Using site-directed mutagenesis, we have now determined that the minimal polypeptide sequence that can confer β-tubulin autoregulation is an amino-terminal MR(E/D)I. Further, although we show that cytoplasmic, endogenous α-tubulin mRNAs are selectively degraded by tubulin-dependent autoregulation, α- and β-tubulin mRNAs are not degraded through a common pathway. Neither the wild-type α-tubulin mRNAs encoded by transfected genes nor α-tubulin mRNAs mutated to encode an amino-terminal MREI yield mRNAs that are autoregulated. The collective findings show that, despite similarities, α- and β-tubulin utilize divergent determinants to link RNA instability to tubulin subunit concentrations.

MATERIALS AND METHODS

Cell culture and transfection. Mouse L cells were maintained in Dulbecco's modified Eagle's medium (4,500 mg/liter of glucose) with 290 mg of glutamine per liter and 10% fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.); CHO cells were maintained in the same medium further supplemented with 0.2 μM proline. Subconfluent L cells were transiently transfected by a modified DEAE-dextran-dimethyl sulfoxide shock procedure described by Lopata et al. (23). CHO cells were used in all transfections of α-tubulin constructs. CHO cells were transfected with supercoiled plasmid DNA for 4 h and then underwent a 2-min 10% glycerol shock step (using a modification of the calcium phosphate precipitation method of Graham and van der Eb [16]).

For all transient transfections, two 100-mm-diameter dishes of subconfluent CHO cells were cotransfected with 10 μg of test construct and 10 μg of pRSVneo (8 μg each per dish of L cells). Eighteen hours after transfection, the two dishes were trypsinized, pooled, and replated in three dishes. Thirty-eight to forty hours posttransfection, medium (with or without 10 μM colchicine [Sigma] or 10 μg of taxol [Calbiochem] per ml) was added. Cells were harvested 5 h later. In experiments with protein synthesis inhibitors, 5 μg of cycloheximide (Sigma) per ml or 200 μg of puromycin (Sigma) per ml was added along with the colchicine. For RNA half-life measurements, transcription was inhibited by addition of 5 μg of actinomycin D (Sigma) per ml.

To obtain stably transfected cell lines, 24 h after application of the DNA, CHO cells were cultured in complete media supplemented with 400 μg of G418 (Gibco-BRL) per ml. The G418-resistant colonies that appeared were picked and expanded in selective media.

Polysome distribution analysis. Polysome distributions in cytoplasmic extracts were analyzed essentially as described before (25, 27). For each β-tubulin mutant tested, two 100-mm-diameter dishes of L cells, transfected as described above, were fed with either normal growth media or media supplemented with 10 μM colchicine for 5 h. For analysis of α-tubulin constructs, three dishes of CHO cells transfected as described above were harvested by trypsinization, pooled, and replated in four dishes. At 38 to 40 h posttransfection, two dishes were fed with normal growth media and the other two were fed with media supplemented with 10 μM colchicine. Five hours later, cytoplasmic extracts were prepared and sedimented on 12-ml 15 to 45% sucrose gradients at 38,000 × *g* for 90 min in a Beckman SW41 rotor. Gradients were fractionated into 16 fractions with continuous monitoring of A_{254}. Equivalent portions of RNA from each gradient fraction were analyzed by S1 nuclease protection for the distribution of mRNA from transfected and endogenous genes.

RNA isolation and S1 nuclease protection analysis. Cytoplasmic RNA was isolated by a modification of the method of Favaloro et al. (12). For hybridization and S1 nuclease protection, 0.01 pmol of each 5′-end-labeled double-stranded DNA probe was mixed in combinations as indicated, hybridized with 5 μg of cytoplasmic RNA for 16 h at 55°C, and then digested with S1 nuclease essentially as described by Yen et al. (36). The protected fragments were resolved on 5% acrylamide–8 M urea gels and visualized by autoradiography. Phosphorimaging was used as indicated for quantitation of RNA levels. In other experiments, densitometry of autoradiographs was used for quantitation.

To distinguish various α- and β-tubulin mRNAs transcribed from endogenous and transfected genes (all linearized probes were dephosphorylated with calf intestinal alkaline phosphatase [BRL] and 5′ end labeled with T4 polynucleotide kinase [New England Biolabs] and [γ-^{32}P]ATP), the following DNA probes were used.

(i) Human β-tubulin encoded by the M40 gene. To detect the mutant human βI-tubulin mRNAs, a human βI cDNA clone (pHβ1) (32) was digested with *Bgl*II, and the plasmid fragment containing the 5′ untranslated region and the coding sequence through the third exon was 5′ end labeled. A 355-nucleotide (nt) fragment of this probe was protected by hybridization with each of the M40 point mutants. For RNAs from pmycM40, an additional 250-nt protected fragment representing partial S1 cleavage at the site of the *myc* sequence insertion was also produced.

(ii) Neo mRNA. The neomycin phosphotransferase gene from pSV2neo (30) was subcloned as a *Bgl*II-to-*Bam*HI fragment into the *Bam*HI site in pGEM1 (Promega). An S1 nuclease probe, generated by linearizing this subclone (p0neo) at the unique *Nco*I site, protected a fragment of 597 nt spanning the 5′ untranslated region and most of the *neo*-coding region. This probe was used as an internal control for transfection efficiency and RNA recovery.

(iii) Endogenous mouse β-tubulin. To detect mRNA from the endogenous mouse mβ5 gene, a probe was prepared from a pUC9-mβ5 cDNA subclone (32). The cDNA was digested with *Asp* 718 and end labeled, and the fragment containing the 5′ untranslated region of the cDNA was isolated for use as a probe. S1 nuclease analysis of mβ5 mRNAs yielded a 109-nt protected fragment.

(iv) SV40-promoted α- and β-tubulin cDNA constructs. pSV40-αT and pSV40-βT were cleaved with *Xma*I and *Bgl*II, respectively, and 5′ end labeled to produce probes that protected mRNA sequences corresponding to the tubulin amino-terminal coding sequences and 5′ untranslated regions, including ~53 bases of transcribed simian virus 40 (SV40) sequences from the cap site of the SV40 early promoter. The probe from pSV40-αT protected both the transfected (SVα [295-nt]) (see Fig. 3) and endogenous (Endα [191-nt]) (see Fig. 3) α-tubulin mRNAs. The probe from pSV40-βT did not cross-react with endogenous β-tubulin mRNAs.

(v) Endogenous hamster β-tubulin. To detect the endogenous β-tubulin mRNAs in CHO cells, two different probes were used. In two experiments, a CHO β-tubulin cDNA (28) was labeled at the unique *Bgl*II site that lies within the protein-coding region. This probe protected βI-tubulin mRNA (Endβ [365 bases]) (see Fig. 3C and 8). In other experiments,

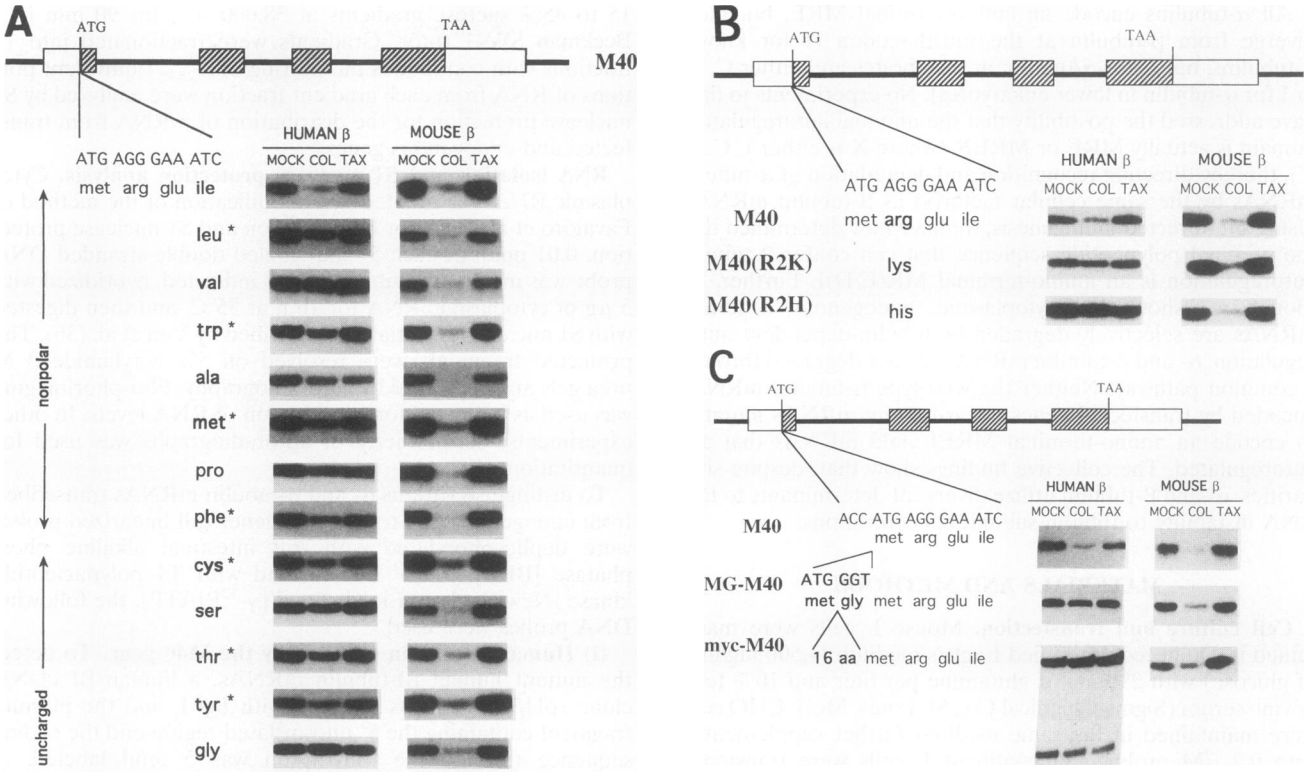

FIG. 1. β-Tubulin autoregulation requires an amino-terminal MR(E/D)I. (A) Autoregulated instability of β-tubulin mRNAs requires isoleucine at codon 4. Schematic diagram of the human β-tubulin gene (M40) with the indicated amino acid substitutions at codon 4. Hatched boxes, coding sequences; open boxes, 5′ and 3′ untranslated sequences; lines, intronic and flanking regions. Below the gene, the fourth-codon mutant amino acids are grouped by charge. Equal amounts of cytoplasmic RNA from control (MOCK), colchicine-treated (COL), and taxol-treated (TAX) transiently transfected L cells were analyzed simultaneously by S1 nuclease protection for endogenous mouse β-tubulin mRNAs (mouse β) and the transfected mutant M40 gene-derived mRNAs (human β). Asterisks indicate mutants retaining partial regulation. (B) Conservative amino acid substitutions (histidine and lysine) at the second codon do not confer autoregulation. M40 β-tubulin genes encoding lysine or histidine at codon 2 were transfected, and accumulated RNAs were analyzed as for panel A. (C) β-Tubulin autoregulation requires the tetrapeptide signal at the extreme amino terminus. Schematic diagram of the M40 human β-tubulin gene with the wild-type amino-terminal sequence shown in the top line and the two progressively larger insertion mutations indicated below. pMG-M40 and pmycM40 were transiently transfected and analyzed as in panel A. Note that pmycM40-encoded mRNAs yielded two protected fragments because of partial cleavage of the probe at the site of the insertion.

an *Apa*LI-*Bam*HI fragment was excised from the CHO β-tubulin cDNA (containing sequences from codon 5 to codon 85) and subcloned into pGEM2. This plasmid was end labeled at the unique *Bgl*II site within the β-tubulin open reading frame and gave rise to a protected fragment of 240 nt.

(vi) Human type I (αI) α-tubulin. An *Nco*I-*Sma*I fragment from pCMV5′α (a cloning intermediate generated in the construction of pCMVkα1; see below) including the 3′ half of the cytomegalovirus (CMV) promoter and the 5′ cDNA PCR fragment was subcloned into pGEM7Z (Promega). This probe was designed to detect mRNAs encoded by the transfected gene and initiated at the expected transcription initiation site. Moreover, the probe also distinguished these RNAs from endogenous α-tubulin mRNAs and any potential aberrant transgene-encoded RNAs derived from transcription initiation at upstream sites. The predicted protected fragment sizes are 450 nt for RNAs initiated more than 170 bases upstream of the expected transcription initiation site, 280 nt for human αI-tubulin mRNA encoded by the transgene and initiated at +1, and 270 nt for endogenous CHO αI-tubulin mRNA (see Fig. 4 for diagram).

(vii) Rat type II (αII) α-tubulin. For mRNAs encoded by the rat αII-tubulin gene tagged in exon 4 with a *Not*I linker (pαTNot), a 5′-end-labeled probe was prepared from a 2.6-kb *Nco*I fragment. Because of the 12-nt insertion, this probe distinguished the transfected rat αII-tubulin mRNA (560 nt) from the endogenous CHO cell αII-tubulin mRNA (450 nt). Endogenous CHO cell isotype I and III α-tubulin mRNAs (which diverge from type II at sequences in the fourth exon [11]) were also detected as a smaller series of ~230-nt fragments (see Fig. 6).

Construction of plasmids. (i) Mutagenesis of the human β-tubulin gene M40. Point mutations were introduced into the fourth and second codons of the human β-tubulin gene

(pM40) (20) by site-directed mutagenesis. A 2.5-kb *Hin*dIII-to-*Bgl*II fragment containing the promoter through the first intron of the M40 gene was subcloned into M13mp18 to prepare a single-stranded template for mutagenesis. To introduce all 19 amino acid substitutions at codon 4, degenerate oligonucleotides [5′-ATGAGGGAANNCGTGCACATC-3′ and 5′-ATGAGGGAAN(G/A/T)GGTGCACATC-3′] were used with the mutagenesis kit from Amersham. To make mutations at codon 2, the M13 subclone of M40 was mutagenized with the degenerate oligonucleotide 5′-TTTAAC ATGG(A/C)A(T/G)GAAATCGTG-3′. This yielded pM40 (R2H) and pM40(R2K) encoding MHEI and MKEI, respectively, at their amino termini. To place a 2-amino-acid (MG) insertion just prior to the normal β-tubulin methionine initiation codon, 5′-ATTTTAACCATGGGTATGAGGGAAAT CG-3′ was used for mutagenesis (this created pATG-M40).

Successful construction of each mutant was confirmed by sequencing. To recreate the full-length human β-tubulin gene from each M13 mutant, a 0.9-kb *Hin*dIII-to-*Nhe*I fragment containing the promoter, the first exon, and 50 bp of the first intron of each mutant was subcloned back into pM40. The presence of the mutation was again reconfirmed by sequencing the final plasmid.

An amino-terminal 18-amino-acid insertion mutant, pmyc M40, was constructed by cloning an epitope from c-*myc* (MEQ KLISEEDLNSCSP) plus two codons from polylinker sequences into the *Nco*I site of pATG-M40 by a three-step strategy. First, a *Hin*dIII-to-*Nco*I fragment containing the promoter and 5′ untranslated region up to the initiator methionine of pATG-M40 was cloned into pMYC-0 (35) digested with the same enzymes. This intermediate was linearized by digestion at the *Pst*I site in the 3′ polylinker (blunted with T7 polymerase) and ligated to a 1.3-kb *Nco*I-to-*Bam*HI fragment (blunted with Klenow polymerase) containing most of exon 1 and the first intron of M40. This second intermediate was digested with *Hin*dIII and *Nhe*I and subcloned back into pM40 as described above (see Fig. 1C).

(ii) α-Tubulin constructs. (a) pSV40-αT and pSV40-βT. A rat α-tubulin cDNA (containing 16 bases of the 5′ untranslated region and the entire coding and 3′ untranslated regions) (21) and a human β-tubulin cDNA (containing the entire 5′ untranslated region and coding region) (20) were subcloned into a derivative of the mammalian expression vector pMSXND (29). The final constructs, pSV40-αT and pSV40-βT, respectively (see Fig. 3), contain the SV40 promoter/enhancer, followed by the cDNA sequence and the SV40 small t-antigen intron and polyadenylation site. Also included in each plasmid is the neomycin phosphotransferase gene which confers resistance to the neomycin analog G418.

(b) pCMVKα1. A human type I α-tubulin cDNA clone, pKα1 (9), was originally cloned by GC tailing and ligation into the *Pst*I site of pBR322. To remove the 5′ GC tail, the 5′ half of the cDNA was amplified by PCR using oligonucleotides HA1 (5′-ATCCTCGAGTGTCGGGGACGGTAACCGG-3′) and HA2 (5′-GACTGTGGGTTCCAAGTCTA-3′). The amplified fragment was double digested with *Xho*I and *Xma*I and cloned in a three-way ligation with the CMV promoter (an *Eco*RI-*Xho*I fragment) and an *Eco*RI-*Xma*I fragment containing the SV40 splice and polyadenylation signals (*Xma*I to *Bam*HI from pSV2neo [30]) cloned into pGEM3 (Promega). This intermediate, pCMV5′α, was digested with *Xma*I and *Hpa*I to remove the *neo*-coding sequence and SV40 splice site, and the 3′ half of the human α-tubulin cDNA (contained on an *Xma*I-*Ssp*I fragment) was restored. The final construct (see Fig. 1) encodes an mRNA transcript that diverges from the mature wild-type human type I α-tubulin only at the first 10 nt in the 5′ untranslated region and the final 60 nt just 5′ to the polyadenylation site.

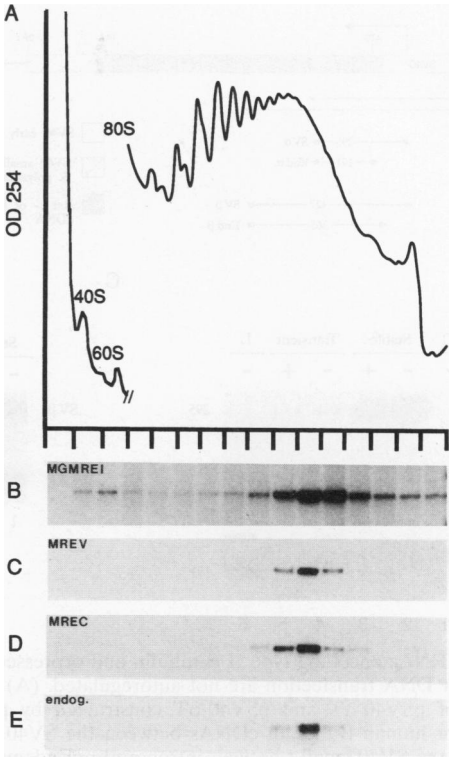

FIG. 2. RNAs from mutant human β-tubulin genes are efficiently translated. (A) Representative polysome profile from lysates of L cells transiently transfected with human β-tubulin mutants (all profiles were essentially superimposable). The 40S and 60S ribosomal subunits and the 80S monosome peaks are indicated. The break in the tracing indicates a twofold change in scale. Sixteen fractions were collected from each gradient and analyzed by S1 nuclease protection for both transfected mutant β-tubulin mRNAs. OD254, optical density at 254 nm. (B) pMG-M40; (C) pM40(I4V); (D) pM40(I4C); (E) endogenous mβ5-tubulin.

(c) pαTNot. The rat type II α-tubulin gene, pRatα, was obtained from P. Sharp and I. Lemischka as a subclone of λαT14 (22) cloned with *Xho*I linkers into the *Pvu*II site of pBR322. In order to allow simultaneous discrimination between the endogenous αI- and αII-tubulin mRNAs and the RNAs derived from this rat α-tubulin gene, 12 nt were inserted at codon 159. To do this, the rat α-tubulin gene was subcloned as an *Xho*I fragment into *Sal*I-digested pGEM1 (destroying the vector *Sal*I site). The subcloned gene was linearized at the unique *Sal*I site in the fourth exon, which was blunt-ended with Klenow polymerase, and a *Not*I linker (GCGGCCGC) was inserted to create four additional codons (see Fig. 6).

(d) pαTNot(C4I). To construct pαTNot(C4I), a human type I α-tubulin gene in which the fourth codon was changed from TGT (cysteine) to ATC (isoleucine), a 1.4-kb *Bam*HI-*Sst*I fragment corresponding to a portion of exon 2 and intron 1 was subcloned into M13mp19 for single-stranded mutagenesis (using oligonucleotide 5′-GGATGGAGATAATCTCACGCTGTG-3′).

RESULTS

The full range of β-tubulin autoregulation requires the consensus tetrapeptide MR(E/D)I. Previous efforts have shown that a signal sufficient for conferring tubulin-dependent

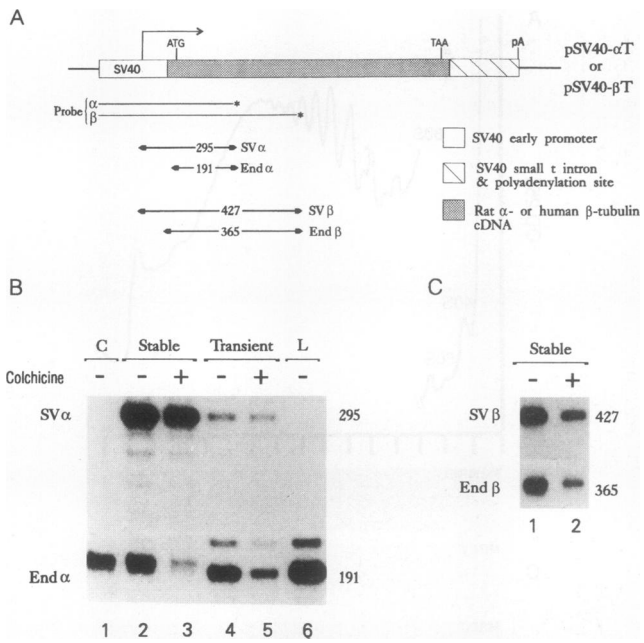

FIG. 3. RNAs encoding type II α-tubulin and expressed by stable or transient DNA transfection are not autoregulated. (A) Schematic drawings of pSV40-αT and pSV40-βT constructed by placing rat α-tubulin or human β-tubulin cDNAs between the SV40 early promoter and the SV40 small t-antigen intron and polyadenylation site. Shown below the diagram are the DNA fragments used as probes for S1 nuclease analysis. The sizes of the fragments protected by the endogenous (Endα and Endβ) or transgene (SVα and SVβ)-encoded α- and β-tubulin mRNAs are indicated in nucleotides for all panels. Levels of RNAs from pSV40-αT (B) and pSV40-βT (C) following stable or transient transfection. The SV40-promoted α- and β-tubulin cDNAs were cotransfected into CHO cells, and colonies expressing both pSV40-αT and pSV40-βT were expanded. A representative stable cell line was treated with colchicine (lane 3) or left untreated (lane 2), and cytoplasmic RNA was analyzed. Lane 1, RNA from untransfected CHO cells. Lanes 4 and 5, pSV40-αT RNA levels in mouse L cells transiently transfected and cultured for the final 3 h in normal media (lane 4) or media containing colchicine (lane 5). Lane 6, Mock transfected L cells. (C) The same RNA samples as in lanes 2 and 3 of panel B were examined by S1 nuclease analysis for the abundance of endogenous (Endβ) and transfected (SVβ) gene-encoded β-tubulin RNAs.

autoregulation on other mRNAs is contained in the first four β-tubulin codons (36, 37). To determine the sequence requirements for this peptide signal, site-directed mutagenesis was used to examine the influence of the amino acid encoded at position four of the β-tubulin nascent peptide. For this, the isoleucine normally encoded at the fourth codon of the human βI-tubulin gene, M40, was converted to all 19 other amino acids (Fig. 1A). The wild-type M40 gene and each mutant were transiently transfected into duplicate dishes of mouse L cells. To control for transfection efficiency and RNA recovery, cells were trypsinized 18 h after transfection, pooled, and replated in three parallel dishes. S1 nuclease analysis was used to measure the level of mRNA accumulated for each transfected gene and endogenous β-tubulin mRNA, both in cells with normal tubulin levels and after colchicine-induced microtubule disassembly or complete assembly of intracellular tubulin by the addition of taxol. Both the endogenous and the transfected wild-type human β-tubulin mRNA levels were diminished following colchicine-dependent elevation of tubulin subunit levels (Fig. 1A), as seen in earlier studies (36, 37). Taxol stimulation of complete microtubule assembly, on the other hand, selectively increased β-tubulin mRNA levels. In each of three independent transfection experiments, 12 of the 19 amino acid substitutions at codon 4 yielded mRNAs that were insensitive to changes in the unassembled tubulin subunit concentrations. Seven substitutions to nonpolar amino acids reproducibly yielded mRNAs that showed partial sensitivity to elevated tubulin subunit levels (denoted with asterisks in Fig. 1A). However, only the wild-type isoleucine yielded the full range of autoregulation seen with the endogenous β-tubulin mRNAs.

Beyond the requirement for isoleucine at position four, to identify the minimal sequence requirement for the tetrapeptide that specifies tubulin-dependent autoregulation of β-tubulin mRNAs, we also examined the influence of conservative amino acid substitutions at codon 2. Previous efforts had shown that all six arginine codons conferred autoregulation while codons for seven hydrophobic or acidic residues did not (37). To examine whether arginine was specifically required or whether other basic amino acids would substitute, we changed codon 2 to either lysine or histidine (Fig. 1B). Neither of these conservative amino acid substitutions retained the ability to signal autoregulation.

In conjunction with earlier efforts that showed that the wild-type glutamate (or the conservative replacement aspartate) was required at codon 3, these collective efforts define the tetrapeptide sequence that can confer cotranslational, tubulin-dependent mRNA instability as MR(E/D)I.

β-Tubulin autoregulation requires the MREI tetrapeptide at the extreme amino terminus of the nascent polypeptide. In its normal context, the tetrapeptide sequence that confers β-tubulin autoregulation lies at the extreme amino terminus of the nascent polypeptide. While one previous effort had shown that placement of the tetrapeptide in the middle of thymidine kinase (103 amino acids from the amino terminus) yielded an mRNA that was not autoregulated (37), to test more directly whether an amino-terminal position was essential for autoregulation, β-tubulin genes containing 2 and 18 additional codons at the extreme amino terminus were constructed. Insertion of a new translation-initiating methionine codon followed by 17 additional codons (e.g., the *c-myc* epitope tag [35]) into an otherwise wild-type M40 gene (producing pmycM40) blocked autoregulation of the mutant mRNAs (Fig. 1C). Even the addition of only two amino-terminal codons (Met-Gly) into an otherwise wild-type β-tubulin mRNA completely abrogated autoregulation (pMG-M40) (Fig. 1C). A trivial explanation for the absence of autoregulation of these two hybrid mRNAs was that the mutant mRNAs were not well translated as a consequence of inserting new putative translation initiation codons. This possibility was demonstrated to be false by examining the positions of pMG-M40-encoded RNAs in polysome gradients (Fig. 2). Despite the complete absence of autoregulation, the β-tubulin RNAs encoding an amino-terminal MGMREI cosedimented with the same high-molecular-weight polyribosomes, as did the endogenous β-tubulin mRNAs (encoding an amino-terminal MREI), indicative of efficient translation. Moreover, two mutant β-tubulins that were not autoregulated as a result of fourth-codon substitutions (e.g., MREV and MREC) were also seen to be as efficiently translated as the endogenous β-tubulin mRNAs (Fig. 2).

Searching for determinants of α-tubulin mRNA autoregulation: RNAs transcribed from hybrid genes expressing type I (αI) or type II (αII) α-tubulin cDNAs are not autoregulated. Since the α-tubulin amino-terminal tetrapeptide MREC did not confer full autoregulated instability in the context of a

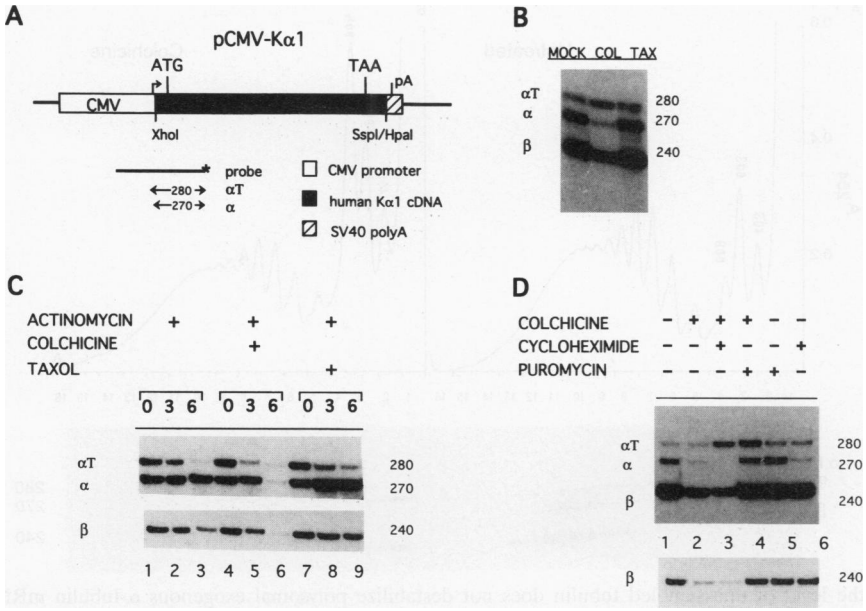

FIG. 4. Human type I α-tubulin transgene mRNA half-life is unaffected by changes in unassembled tubulin concentration. (A) Schematic representation of pCMVkα1. The DNA probe used for S1 nuclease mapping is shown along with the 270-nt fragment protected by the endogenous α-tubulin mRNA (α) and the 280-nt fragment protected by the transfected α-tubulin mRNA (αT). polyA (pA), the SV40 polyadenylation signal and 3′ flanking sequences from pSV2neo. (B) S1 nuclease analysis of tubulin mRNA levels from cells treated with antimicrotubule drugs. CHO cells, transiently transfected with pCMVkα1, were treated for 5 h with media alone (MOCK), colchicine (COL), or taxol (TAX), and endogenous and transfected RNAs were detected by S1 nuclease mapping. The probe, diagrammed in panel A, detected both the endogenous type I α-tubulin (α; 270-nt) and the transfected α-tubulin (αT; 280-nt) mRNAs. A probe for the endogenous β-tubulin was also included in each S1 nuclease analysis; this protected a 240-nt fragment. (C) Half-life determinations for tubulin mRNAs encoded by endogenous and transfected genes. CHO cells were transiently transfected with pCMVkα1 and then treated for 0, 3, or 6 h with actinomycin D alone (lanes 1 to 3) or with actinomycin D plus colchicine (lanes 4 to 6) or taxol (lanes 7 to 9). Equal amounts of cytoplasmic RNA were analyzed by S1 nuclease mapping for transfected α-tubulin (αT) and the endogenous α- and β-tubulin transcripts (α and β). (D) Effect of inhibiting translation elongation on tubulin autoregulation. CHO cells transiently transfected with pCMVkα1 were treated for 5 h with fresh medium (lane 1), medium with colchicine alone (lane 2), colchicine and cycloheximide (lane 3), colchicine and puromycin (lane 4), puromycin alone (lane 5), or cycloheximide alone (lane 6). Transfected (αT) and endogenous (α and β) tubulin mRNAs were detected by S1 nuclease protection analysis of cytoplasmic RNA. A shorter exposure of the endogenous β-tubulin signals is shown in the lower panel.

β-tubulin mRNA (Fig. 1A), we next examined directly whether α-tubulin mRNA stability is autoregulated by the same mechanism as that of β-tubulin. To do this, we initially prepared hybrid α-tubulin genes carrying either nearly full-length cDNA sequences for the rat type II α-tubulin (21) or the ubiquitously expressed human type I α-tubulin (9). For the first of these, an analogous pair of genes was built with the rat αII-tubulin (pSV40-αT) or human βI-tubulin (pSV40-βT) cDNAs inserted between the SV40 early promoter and the small t-antigen splice and polyadenylation sites (Fig. 3). These were transiently transfected into L cells or stably cotransfected into CHO cells, and cloned lines were established. The levels of the transgene-encoded rat αII-tubulin mRNA (Fig. 3B) and β-tubulin mRNA (Fig. 3C) were determined by S1 nuclease analysis both before and after colchicine-mediated microtubule disassembly. Although endogenous α-tubulin mRNAs were reduced more than fivefold by microtubule disassembly, transgene-encoded rat α-tubulin mRNAs were only slightly (if at all) diminished following stable transfection into hamster cells (SVα; Fig. 3B, lanes 2 and 3) or transient transfection into mouse L cells (SVα; Fig. 3B, lanes 4 and 5). On the other hand, following stable (Fig. 3C) and transient (not shown) transfection, endogenous β-tubulin mRNAs and those encoded by the SV40-promoted β-tubulin cDNA were destabilized to the same extent in response to elevation of the unassembled tubulin subunit level (Fig. 3C).

In the second α-tubulin cDNA construct, a nearly full-length human αI-tubulin cDNA was placed under the control of the CMV promoter. In the actual construct, the predicted RNA transcript included 10 bases of sequence from CMV, 68 bases of the α-tubulin 5′ untranslated region, the complete α-tubulin-coding sequence, and 130 of 168 bases of the α-tubulin 3′ untranslated region followed by the SV40 small t-antigen polyadenylation site (Fig. 4). The final construct, pCMVkα1, was transiently transfected into parallel dishes of CHO cells, and cells from sister dishes were pooled and replated to eliminate variations in transfection efficiency. Thirty-six hours after transfection, colchicine or taxol was added for 5 h to elevate or deplete the pool of unassembled tubulin subunits. Equivalent amounts of cytoplasmic RNA from treated and untreated cells were analyzed in parallel by using S1 nuclease protection with probes that recognized the transfected α-tubulin, endogenous α-tubulin, and endogenous β-tubulin mRNAs. As seen before, the levels of both endogenous α- and β-tubulin mRNAs declined about fivefold when the unassembled tubulin subunit concentration was elevated following colchicine-mediated microtubule disassembly (Fig. 4B). Unlike the situation for β-tubulin RNAs expressed from similar hybrid genes (Fig. 1 and 3) (14, 25, 36), the levels of an mRNA carrying the full coding region and most of the 5′ and 3′ untranslated sequences of a human αI-tubulin mRNA were not affected by changes in the assembly state of the microtubules (the αT bands in Fig. 4B).

Autoregulation of α-tubulin mRNAs is mediated by cyto-

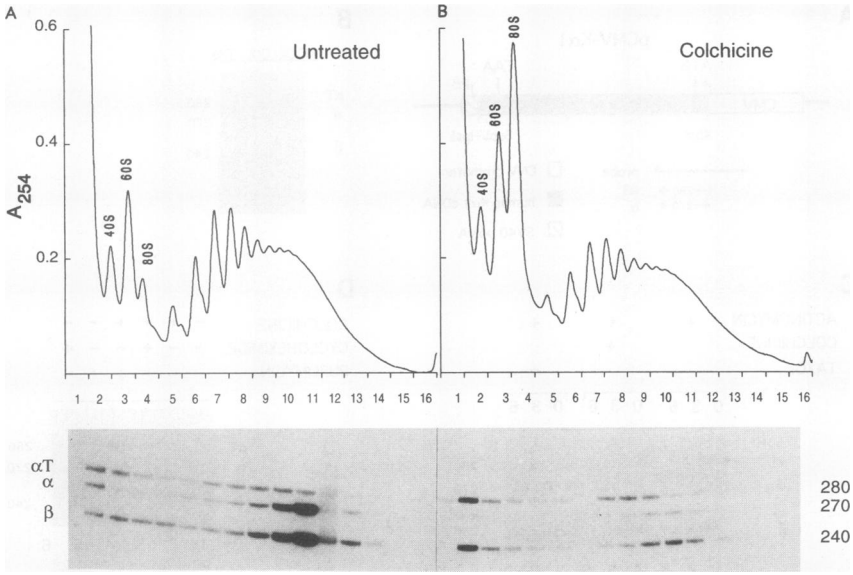

FIG. 5. Elevation of the level of unassembled tubulin does not destabilize polysomal exogenous α-tubulin mRNA. CHO cells transiently transfected with pCMVkα1 were incubated in the absence (A) and presence (B) of colchicine for 5 h. Cytoplasmic extracts were prepared and sedimented on 15 to 45% sucrose gradients to analyze the distribution of tubulin mRNAs on polyribosomes. The gradients were collected in 16 fractions, and RNA was extracted from equivalent proportions of each fraction. The top panels show the polysome profile for each gradient. The direction of sedimentation is from left to right. The positions of the 40S and 60S ribosomal subunits and the 80S monomer peak are indicated. The bottom panel shows the distribution of the transfected α-tubulin (αT) and endogenous tubulin mRNAs (α and β) as assessed by S1 nuclease protection (Fig. 4, diagram of probe). Sizes are indicated (in nucleotides) on the right.

plasmic mRNA instability. The absence of autoregulation by these nearly full-length α-tubulin RNAs was unexpected. To examine mRNA half-lives more directly and to rule out the possibility of unexpected (and unwanted) changes in transcriptional activity following colchicine- or taxol-induced changes in microtubule assembly, cells were transfected with pCMVkα1, pooled, and replated 36 h after transfection. Transcription was then blocked by the addition of actinomycin D alone or actinomycin D and colchicine or taxol. Endogenous α- and β-tubulin mRNAs were relatively stable in the cytoplasm after inhibition of transcription (with half-lives of >12 and ~5 h, respectively) (Fig. 4C, lanes 1 to 3), but endogenous α- and β-tubulin mRNAs were significantly destabilized after the tubulin subunit concentration was increased by colchicine-induced microtubule disassembly (Fig. 4C, lanes 4 to 6). Conversely, taxol-induced depletion of the unassembled tubulin pool prevented endogenous α- and β-tubulin mRNAs from decaying detectably within 6 h (Fig. 4C, lanes 7 to 9). Unlike these endogenous tubulin mRNAs, the transfected type I (αI) α-tubulin mRNAs were unaffected by changes in microtubule assembly and displayed a relatively short half-life (~2 h) in the presence of normal, elevated, and depleted tubulin subunit levels (Fig. 4C).

Tubulin autoregulation has previously been reported to be enhanced by slowing (but not completely stopping) ribosome transit in the presence of low concentrations of cycloheximide (13, 25). As an additional test of whether the pCMVkα1-encoded α-tubulin mRNA can be a substrate for the type of autoregulation that typifies β-tubulin, dishes of transiently transfected CHO cells were pooled, replated, and then treated with colchicine alone, colchicine and a low concentration of cycloheximide, or colchicine and puromycin. Again, as expected for the endogenous α- and β-tubulin mRNAs, polysome disruption by puromycin blocked autoregulation (compare Fig. 4D, lanes 2 and 4), while slowing ribosome transit with cycloheximide enhanced autoregulated RNA loss (compare Fig. 4D, lanes 2 and 3). However, unlike the authentic tubulin RNAs, the pCMVkα1-encoded mRNA increased in abundance after the addition of either translation inhibitor (Fig. 4D, lanes 5 and 6), and this apparent RNA stabilization was actually enhanced by elevation of the tubulin subunit level (Fig. 4D, lanes 3 and 4).

Since only well-translated α- or β-tubulin mRNAs are substrates for tubulin-dependent degradation (25, 36), if CMVkα1 mRNA was, for whatever reason, poorly translated, this could explain why it was not a substrate for this autoregulatory mechanism. To determine the translational state of CMVkα1 mRNA, polysome profiles of transfected CHO cells were analyzed. In extracts from cells with normal tubulin subunit levels, ~50% of the CMVkα1 mRNA cosedimented with 7- to 10-mer polysomes (Fig. 5A, lanes 9 and 10), whereas the majority of endogenous α-tubulin mRNAs sedimented with 10- to 14-mer polysomes (lanes 10 and 11), indicative of very efficient translation of the 454-codon mRNA. (The relatively large amount of nonpolysomal mRNAs in this experiment is atypical and probably reflects some ribosome runoff in vitro.) A similar situation was seen after elevation of tubulin subunit levels, except that the abundance of the endogenous α-tubulin mRNAs was markedly diminished (Fig. 5B).

We conclude that, despite relatively efficient translation, the nearly wild-type α-tubulin mRNA encoded by pCMVkα1 is not degraded by the tubulin autoregulatory mechanism.

Searching for determinants of α-tubulin mRNA instability: rat type II α-tubulin mRNAs expressed by transfection are not autoregulated even when they are efficiently translated and encode an amino-terminal MREI. Since the RNA encoded by pCMVkα1 differs from the authentic α-tubulin mRNA at both the extreme 5′ and 3′ termini, the simplest explanation for why this chimeric α-tubulin mRNA was not autoregulated is that one or both of these domains were necessary for tubulin-

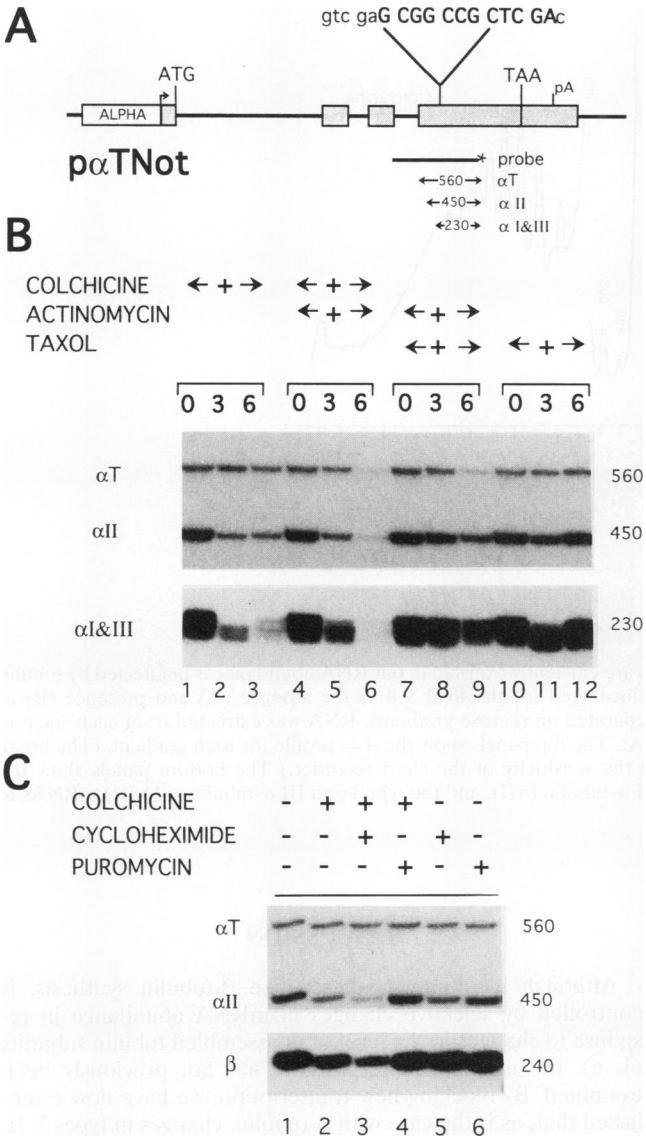

FIG. 6. Accumulated levels of RNAs encoded by a transfected rat α-tubulin gene are not affected by the levels of unassembled tubulin. (A) Schematic diagram of pαTNot. The rat type II α-tubulin gene was modified by insertion of a *Not*I linker (the sequence is indicated by the uppercase lettering above the drawing) to create pαTNot. Open box, rat α-tubulin promoter; shaded boxes, exons; heavy lines, introns and flanking sequences. The translation initiation and termination codons and the polyadenylation site (pA) are also marked. A 5′-end-labeled probe was used to distinguish among the transfected (αT) and endogenous αII-tubulin (aII) mRNAs and α-tubulin mRNAs encoding isotypes I and III (αI&III). Sizes (in nucleotides) of the protected fragments are indicated within the arrows (A) or are shown on the right (B and C). (B) Stability of mRNAs encoded by pαTNot is not autoregulated. CHO cells were transiently transfected with pαTNot and then treated for 3 or 6 h with colchicine (lanes 2 and 3), colchicine and actinomycin D (lanes 5 and 6), taxol and actinomycin D (lanes 8 and 9), or taxol (lanes 11 and 12) or harvested untreated (lanes 1, 4, 7, and 10). The time of drug treatment is indicated in hours above each lane. The levels of transfected (αT) and endogenous α-tubulin mRNAs (αII and αI & III) were analyzed by S1 nuclease mapping. (C) Levels of α-tubulin RNAs encoded by pαTNot are not altered by disruption of translation. CHO cells were transiently transfected with pαTNot and treated for 4 h with fresh medium (lane 1), medium with colchicine alone (lane 2), colchicine and a low level of cycloheximide (lane 3), colchicine and puromycin (lane 4), cycloheximide alone (lane 5), or puromycin alone (lane 6). Transfected (αT) and endogenous type II (αII) α-tubulin and endogenous β-tubulin (β) mRNAs were detected by S1 nuclease protection of cytoplasmic RNA isolated from the drug-treated cells.

dependent destabilization. To test this, an authentic rat αII-tubulin gene (including the promoter and 3′ downstream sequences) was transfected, and the behavior of the encoded mRNA was examined before and after elevation of tubulin subunit levels. To allow discrimination between the transfected and endogenous α-tubulin mRNAs, an additional four codons were added to the fourth exon of the rat gene by insertion of 12 nt at codon 159 (producing gene construct pαTNot). A 5′-end-labeled S1 probe that quantitatively distinguishes the type II α-tubulin mRNA encoded by the transfected gene (560-nt predicted product) (Fig. 6A) from the endogenous type II α-tubulin mRNA (450-nt predicted product) and the more abundant endogenous type I and III α-tubulin mRNAs (230-nt predicted products) was prepared. All three endogenous α-tubulin mRNAs diminished in abundance following colchicine-induced microtubule disassembly (Fig. 6B, lanes 1 to 3). Simultaneous inhibition of transcription with actinomycin D proved that mRNA loss was the result of a decrease in cytoplasmic mRNA half-life (Fig. 6B, lanes 4 to 6). (The pool of nuclear α-tubulin mRNAs in these cells is less than 5% of the cytoplasmic level [8]). A small stabilization of each endogenous mRNA was seen after taxol-induced depletion of the tubulin subunit pool (Fig. 6B, lanes 7 to 12). In contrast, although the transgene encodes a fully wild-type α-tubulin mRNA (except for the linker insertion), the abundance and apparent half-life of the α-tubulin mRNA transcribed from pαTNot were unaffected by changes in subunit concentration (compare lanes 4 to 6 with lanes 7 to 9 of Fig. 6B).

As a more sensitive test for whether the rat type II α-tubulin mRNA encoded by pαTNot can be a substrate for autoregulation in transiently transfected rodent cells, translation inhibitors were added along with the microtubule inhibitory agents and the effects on steady-state levels of the mRNAs encoded by the transfected and endogenous genes were examined. Although, as before, colchicine-dependent loss of endogenous αII-tubulin mRNAs was stimulated by slowing translation elongation with cycloheximide (Fig. 6C, lanes 2 and 3), this did not affect RNA levels from the transfected α-tubulin gene (lane 3). Ribosome dissociation following addition of puromycin (Fig. 6C, lane 4) also had no effect on the levels of pαTNot-encoded mRNAs, although the endogenous α- and β-tubulin mRNA levels rose as disruption of ribosomes blocked autoregulated mRNA degradation (lanes 4 and 6).

To ensure that the 454-codon rat type II α-tubulin mRNA encoded by the transfected gene and each of the endogenous mRNAs encoding the 449- to 451-amino-acid CHO α-tubulin isotypes were efficiently translated, polysomes were analyzed from both untreated and colchicine-treated CHO cells 36 h after transfection with pαTNot. The rat type II α-tubulin sedimented primarily with 10- to 14-mer polysomes, as did the efficiently translated endogenous α-tubulin mRNAs (Fig. 7A). Following colchicine-induced elevation in the tubulin subunit concentration, polysomal levels of all three isotypes of endogenous α-tubulin mRNAs were diminished in relative abundance (compare the relative peak intensities of the untreated and colchicine-treated endogenous αI, αII, and III α-tubulin mRNAs in Fig. 7), whereas the transgene-encoded mRNA (designated αT) remained unaffected in abundance and position in the polysome gradient. We conclude that, in contrast to

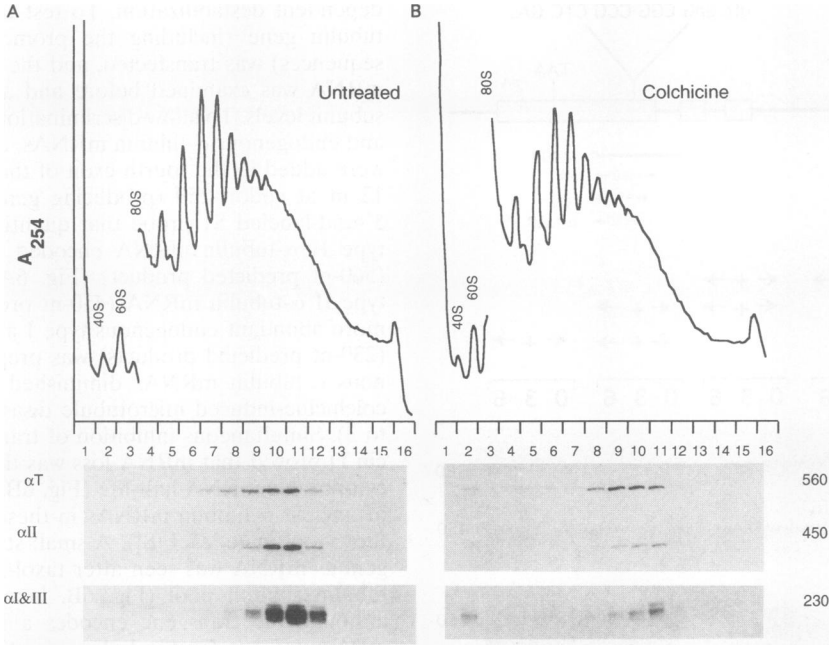

FIG. 7. Cytoplasmic rat α-tubulin mRNAs encoded by transfected genes are efficiently translated, but RNA abundance is unaffected by tubulin subunit levels. CHO cells were transiently transfected with pαTNot and incubated for the final 5 h in the absence (A) and presence (B) of colchicine. Cytoplasmic extracts were prepared, and polyribosomes were separated on sucrose gradients. RNA was extracted from each fraction and analyzed by S1 nuclease protection for the presence of α-tubulin mRNAs. The top panels show the A_{254} profile for each gradient. (The break in the absorbance profile at the 80S peak is due to a twofold change in the sensitivity of the chart recorder.) The bottom panels show the sedimentation of the transfected rat α-tubulin (αT), the endogenous type II α-tubulin (αII), and the type I and III α-tubulin (αI&III) mRNAs in each gradient (Fig. 6, diagram of S1 nuclease probe).

RNAs from β-tubulin genes, wild-type rat αII tubulin mRNAs expressed by transient transfection are not an efficient substrate for the tubulin autoregulatory mechanism.

In a last effort to examine whether the rat αII-tubulin mRNA expressed by transfection could be converted to a substrate for autoregulation, site-directed mutagenesis was used to alter the encoded amino-terminal polypeptide sequence from the MREC corresponding to α-tubulin to the β-tubulin tetrapeptide MREI. The corresponding gene [pαTNot(C4I)] was transiently cotransfected into L cells along with a neomycin gene that, as before, served as a transfection control. Neither the αII gene modified to encode MREI nor the original gene yielded mRNAs (αT in Fig. 8A) that were efficiently autoregulated, although partial (twofold when quantified by phosphorimaging) autoregulation could be detected by simultaneous microtubule disassembly and slowing of ribosome transit with cycloheximide (Fig. 8A, lanes 2 and 5). As always, endogenous αI, II, and III mRNAs as well as β-tubulin mRNAs were significantly diminished by colchicine-induced microtubule disassembly (Fig. 8A, lanes 3 and 6), an effect accelerated by slowing ribosome transit (lanes 2 and 5). Neomycin RNA levels were not affected by any treatment. Phosphorimaging data (and an identical experiment done independently) confirmed this qualitative result: while slowing ribosome transit did yield twofold diminution of both pαTNot and pαTNot(C4I) α-tubulin RNAs, a much larger, 10-fold effect was measured for each of the endogenous α- and β-tubulin mRNAs. Moreover, the failure of pαTNot- and pαTNot(C4I)-encoded mRNAs to autoregulate efficiently was also apparent in cells stably expressing either gene (Fig. 8B).

DISCUSSION

Although α-tubulin synthesis, like β-tubulin synthesis, is controlled by selective changes in mRNA abundance in response to changes in the level of unassembled tubulin subunits (1, 6), the mechanism responsible has not previously been examined. By blocking new transcription, we have now established that, as is the case with β-tubulin, changes in types I, II, and III α-tubulin mRNA levels are mediated by changes in cytoplasmic mRNA stability in response to elevated unassembled tubulin subunit concentrations. Further, also as with β-tubulin mRNAs, there is a direct involvement of the translating ribosome(s) in the degradation of α-tubulin mRNA since polysomal α-tubulin mRNAs are preferentially lost when tubulin subunit concentrations are elevated (Fig. 5). This confirms earlier observations that subunit-dependent changes in α- and β-tubulin mRNA levels are prevented when polysomes are disrupted by protein synthesis inhibitors (15, 25), whereas slowing ribosome translocation accelerates autoregulated degradation of both α- and β-tubulin mRNAs.

The amino-terminal tetrapeptide of all vertebrate α-tubulins, MREC, is intriguingly similar to the β-tubulin amino-terminal MREI, which is sufficient to confer tubulin-dependent autoregulation on thymidine kinase mRNA (36). Examination of the instability of β-tubulin mRNAs containing all 19 amino acid substitutions at codon 4 indicates that only an amino-terminal MREI can confer the full range of tubulin-dependent autoregulation on RNAs transcribed from transfected genes. Combined with a similar mutagenesis revealing a requirement for arginine at codon 2 (Fig. 1C) and with previous efforts showing either glutamate or aspartate to function at codon 3

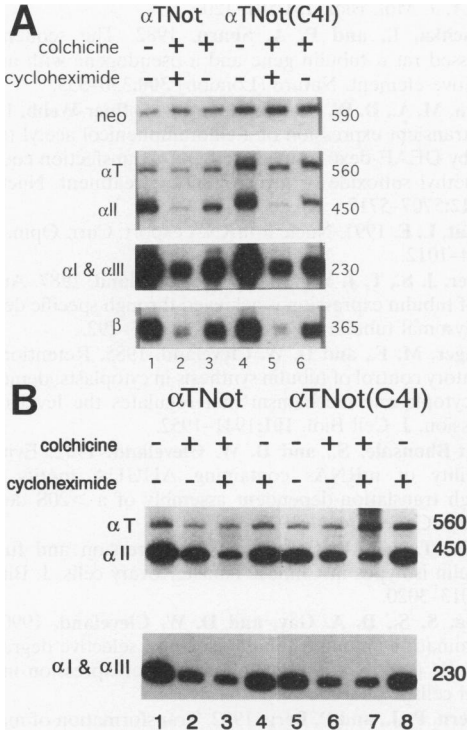

FIG. 8. Nucleotide substitutions to convert the MREC amino-terminal coding sequence of an α-tubulin mRNA into the β-tubulin MREI do not result in efficient autoregulation of α-tubulin mRNAs expressed by transient (A) and stable (B) DNA transfections. (A) Analysis after transient DNA transfection. The rat αII-tubulin gene (pαTNot) and a derivative in which codon 4 was mutated from cysteine to isoleucine [pαTNot(C4I)] were transiently cotransfected with pRSVneo into CHO cells. Accumulated RNAs were analyzed from parallel dishes of cells cultured for the final 5 h in normal media (lanes 1 and 4) or media containing colchicine (lanes 3 and 6) or colchicine and cycloheximide (lanes 2 and 5). Levels of transfected α-tubulin RNAs (αT) endogenous α-tubulins (αI, αII, and IIIα) were analyzed as for Fig. 6. (The probe for endogenous β-tubulin is diagrammed in Fig. 3.) The RNAs from the neomycin phosphotransferase gene (*neo*) were also analyzed simultaneously to ensure comparable efficiencies of transfection and RNA recoveries. (B) Analysis after stable DNA transfection. The plasmids described above were cotransfected into CHO cells along with pRSVneo. After selection with G418, >100 colonies from each transfection were pooled, expanded, and treated for 5 h with normal media (lanes 1 and 5) or media containing colchicine (lanes 2 and 6), colchicine and cycloheximide (lanes 3 and 7), or cycloheximide (lanes 4 and 8). Cells were harvested, and the levels of α-tubulin mRNAs were determined as described for panel A.

(37), this defines a consensus tetrapeptide of MR(E/D)I for β-tubulin autoregulation. Moreover, the MREI domain must also be strictly positioned at the amino terminus since the displacement of this domain by as little as two amino acids disrupts autoregulation.

Our results with hybrid and wild-type α-tubulin genes, combined with the β-tubulin mutagenesis, indicate that the autoregulatory mechanism controlling α-tubulin mRNA decay must be different from that of β-tubulin mRNA. Indeed, a mechanism recognizing a short amino-terminal nascent peptide appears to be specific for β-tubulin alone. Perhaps even more surprising is that no domain(s) on α-tubulin mRNAs can confer efficient autoregulated instability following forced expression using transient or stable DNA transfection. Neither hybrid mRNAs carrying the full-length α-tubulin protein-coding regions fused to heterologous promoter sequences and 5′ and 3′ untranslated sequences nor an essentially wild-type mRNA transcribed from an authentic α-tubulin gene was selectively destabilized when tubulin subunit levels were elevated. Not even conversion of the α-tubulin amino terminus to MREI produced an autoregulated mRNA. Clearly, although the MREI tetrapeptide does confer autoregulated mRNA stability on β-tubulins and when placed as the amino-terminal coding sequence of other mRNAs (e.g., thymidine kinase [36]), other determinants can override MREI-mediated mRNA autoregulation.

If the signal that mediates mRNA degradation is not carried within the mRNA primary sequence or its encoded translation product, where can it reside? One possible explanation is that α-tubulin autoregulation is achieved through cell type- or species-specific regulatory domains in α-tubulin RNAs. Hence, in our experiments, the human and rat α-tubulin mRNAs would not be regulated correctly in the mouse and hamster cells used as recipients for transfection. Although we have not transfected rat or human cells to eliminate possible species specificity, the presence of α-tubulin autoregulation in a wide variety of metazoans (from species as diverse as *Drosophila melanogaster* and humans [6]) argues strongly against this explanation.

A second possibility is that apparent changes in cytoplasmic stability (measured after blocking new transcription with actinomycin D) really reflect changes in the efficiency of maturation and/or transport of nuclear α-tubulin mRNAs transcribed from the endogenous α-tubulin genes. Such a hypothesis seems increasingly plausible in light of strong evidence for nuclear tracking of some mRNAs (18, 19, 24). Hence, after blocking new transcription, changes in cytoplasmic RNA levels could result from changes in the efficiency of maturation of nuclear precursors. But this would require substantial pools of α-tubulin RNAs that could be matured more efficiently when tubulin subunit levels were low, thereby producing higher cytoplasmic mRNA levels and higher apparent cytoplasmic stability. This possibility cannot be correct: nuclear α-tubulin mRNAs are less than 5% of the cytoplasmic level (8), and hence changes in maturation can have only minimal effects on cytoplasmic mRNA levels after blockage of transcription. Moreover, the differential effects of two protein synthesis inhibitors can be readily explained only if the regulation of α-tubulin mRNAs occurs in association with ribosomes, i.e., in the cytoplasm.

Although the mechanism underlying changes in α-tubulin mRNA stability remains unknown, what can be safely concluded is that the mechanism is more complicated than the one that mediates β-tubulin mRNA stability. However, as for many other eukaryotic examples (GM-CSF [27], histones [17], and a series of yeast RNAs [10]), a common feature for both α- and β-tubulin mRNAs is that RNA degradation occurs cotranslationally.

ACKNOWLEDGMENTS

This work has been supported by grant GM29513 from NIH to D.W.C. N.T. has been supported by a postdoctoral fellowship from NIH.

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