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Reconstruction of Appropriate Tubulin and Actin Gene Regulation After Transient Transfection of Cloned β-Tubulin and β-Actin Genes

JOSEPH T. Y. LAU, MARK F. PITTENGER, AND DON W. CLEVELAND*

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

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Most animal cells rapidly depress the synthesis of new α- and β-tubulin polypeptides in response to microtubule inhibitors that increase the pool of depolymerized subunits. This apparent autoregulatory control of tubulin synthesis is achieved through the modulation of tubulin mRNA levels. To begin to analyze the molecular mechanism responsible for such regulation, we have introduced exogenous β-tubulin gene sequences into cultured mouse cells by DEAE-dextran-mediated DNA transfection. We find that the heterologous tubulin genes are expressed and that their RNA transcripts are accurately processed to mature mRNAs. Moreover, after drug-induced microtubule depolymerization, the expression of unintegrated tubulin gene sequences is regulated coordinately with the endogenous mouse α- and β-tubulin RNA transcripts. Such regulation appears to be specific for transfected tubulin genes, since similar down-regulation is not observed in a cotransfected β-actin gene. Curiously, in response to microtubule depolymerization, the amount of RNA transcripts from a transfected β-actin gene increases twofold, which qualitatively and quantitatively parallels that seen by the RNAs encoded by the endogenous actin genes. Thus, the transient DNA transfection approach may permit the unambiguous elucidation of regulatory sequences involved in establishing the proper level of expression of these two important cytoskeletal gene families.

Microtubules, which are comprised principally of dimeric subunits of one α- and one β-tubulin polypeptide, participate in a diverse spectrum of cellular functions, including the establishment of programmed modifications of cell shape during morphogenesis, the formation of mitotic and meiotic spindles, and the establishment of some forms of intracellular and intercellular motility. Given the important functions of these dynamic arrays of microtubules, it is not surprising that the synthesis of tubulin should be a closely regulated process. As initially reported by Ben Ze'ev et al. (1), marked alterations in the morphology of cultured animal cells after drug-induced microtubule depolymerization are accompanied by the specific repression of new tubulin synthesis. What emerged less expectedly from this work and from our own subsequent efforts (7, 8, 10–12) is the realization that the rates of tubulin synthesis are apparently established in these cells by an autoregulatory pathway which is closely linked to the pool size of unpolymerized subunits.

Evidence in support of this autoregulatory model has accumulated from a variety of experiments. The treatment of cells with colchicine or nocadozole, antimicrotubule drugs which induce microtubule depolymerization (for examples, see references 13 and 28) and a concomitant twofold increase in the pool of depolymerized subunits (17, 25), results in a specific (5- to 10-fold) repression of new tubulin synthesis (1, 10). On the other hand, the treatment of cells with vinblastine, an antimicrotubule drug which induces not only microtubule depolymerization but also precipitation of the depolymerized subunits (for an example, see reference 4), yields a mild increase in new tubulin synthesis (1, 10). Moreover, treatment with taxol, a drug that stimulates polymerization and presumably lowers the pool of unpolymerized subunits to a negligible level (22, 23), induces an increase in new tubulin synthesis (10) which under some conditions can be quite dramatic (J. S. Pachter and D. W. Cleveland, unpublished data).

* Corresponding author.

These alterations in tubulin synthetic rates do not simply reflect an inherent cell cycle-dependent program of tubulin synthesis which is uncovered by drug-induced cell cycle disruption. This conclusion arises from three independent lines of evidence. First, the maximum effect is seen within 3 h of drug addition, a time too short to yield a substantial cell cycle blockage in an initially unsynchronized culture. Second, colchicine and nocadozole induce mitotically blocked cell populations which are essentially identical to that induced by vinblastine, even though the effects on tubulin synthetic rates are of opposite signs. Third, in contrast to the dramatic repression of tubulin synthesis induced by colchicine or nocodazole treatment, a simple calculation using the reported threefold decline in the overall rate of protein synthesis during mitosis (14) coupled with the known twofold relative increase in tubulin synthesis at mitosis (3) yields a predicted 6% increase in the relative tubulin synthetic rate after a 3-h drug-induced mitotic arrest for cells with a doubling time of 16 h.

Further support for the autoregulatory model has emerged from the elevation of intracellular tubulin content by direct microinjection of purified tubulin subunits to a level comparable to that which would be liberated by endogenous microtubule depolymerization. After microinjection, tubulin polypeptide synthesis has been found to be rapidly and specifically repressed (11).

Although the molecular mechanism through which this apparent autoregulation is achieved has not yet been identified, we have previously determined that the down-regulation of tubulin synthesis in response to an increased pool size of free subunits is accompanied by a rapid loss of tubulin mRNAs (10). Hence, regulation cannot be achieved through a reversible RNA sequestration mechanism. In addition, the apparent rates of tubulin gene transcription in nuclei isolated from cells with normal or elevated pools of tubulin subunits have been found to be indistinguishable (7). This finding strongly suggests that transcription is not the principal level at which control of tubulin synthesis is exercised. Thus, the

sum of the present data points to a relatively novel autoregulatory control mechanism which is operative at the level of tubulin mRNA processing/transport efficiency or mRNA stability.

To further dissect the precise molecular events which underlie this autoregulation, we have now sought to define the important control sequences that are carried on a tubulin gene or its corresponding mRNA or both. To this end, we have used DNA transfection to transiently introduce a heterologous chicken β-tubulin gene into cultured mouse L cells. We report that the mouse cells correctly express and process the RNA transcript copied from this gene. Moreover, upon elevation of the intracellular pool of unpolymerized tubulin subunits by drug-induced microtubule depolymerization, the level of the transfected chicken tubulin gene RNA transcripts is down-regulated coordinately with the endogenous mouse α- and β-tubulin RNAs. Curiously, at least in mouse L cells, the level of RNAs encoded by the endogenous actin genes is elevated by ca. twofold in response to the antimicrotubule drug colchicine. This up-regulation of actin expression is also reconstituted upon transfection of a chicken β-actin gene into mouse L cells. Hence, the appropriate expression of heterologous tubulin and actin genes can be successfully reconstructed after transfection into cultured cells.

MATERIALS AND METHODS

Recombinant plasmids carrying tubulin or actin sequences. Plasmids pβG1 and pβG2, carrying the complete transcription units of the chicken β1 and β2 tubulin genes, respectively, have been described previously (21). A clone containing the human M40 β-tubulin gene (19) was a kind gift from Nick Cowan, New York University Medical Center. The entire M40 gene contained on a single *Hin*dIII-*Eco*RI fragment (see restriction map of Lee et al. [19]) was subcloned between the *Hin*dIII and *Eco*RI sites of pBR322. We have designated the final construct pM40. pβAct, a plasmid containing the chicken β-actin gene (18) cloned into the *Eco*RI site of pBR322, was kindly provided by Thomas Kost of Norden Laboratories. cDNA plasmids pT1, pT2, and pA1, carrying 92, 99, and 99% of chicken α-tubulin, chicken β2 tubulin, and chicken β-actin mRNA sequences, respectively, have been previously characterized (9, 27).

Cell culturing and transfection protocol. Mouse L cells were routinely maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. One day before transfection, cells were plated onto 100-mm Falcon dishes at a density of 1.8×10^6 cells per dish. The transfection procedure was essentially as described previously by Lopata et al. (20). Briefly, the cells were washed with DMEM without serum, and the medium was replaced with 4 ml of DMEM containing 0.5 mg of DEAE-dextran (M_r of ~500,000; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) per ml and 2.0 μg of DNA per ml. The dishes were returned to a 37°C, 5% CO_2 incubator for 4 h. After this incubation, the medium was removed by aspiration, and the cells were subjected to a brief (3-min) shock with 2.5 ml of 10% dimethyl sulfoxide in HBS (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, 21 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.1]). This 10% dimethyl sulfoxide solution was removed, and the cells were washed with phosphate-buffered saline. Finally, DMEM containing 10% fetal calf serum was added, and the cells were returned to the 37°C, 5% CO_2 incubator. Typically, total cell RNA was prepared from the cells 20 to 26 h after transfection. When appropriate, colchicine was added to the media to a final concentration of 10 μM for a specified period before harvesting the cells. In all cases, RNA was prepared by the guanidine isothiocyanate procedure of Chirgwin et al. (6) coupled with the cesium chloride centrifugation step of Glisin et al. (15).

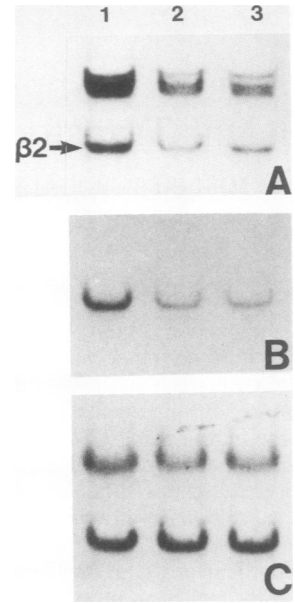

FIG. 1. Modulation of chicken tubulin mRNA levels in response to colchicine-induced microtubule depolymerization. Triplicate dishes of secondary chicken fibroblasts were incubated for 0 h (lane 1), 3 h (lane 2), or 6 h (lane 3) in media containing 10 μM colchicine. Total RNA was prepared, and equal amounts of RNA from each were analyzed by RNA blotting for the presence of β-tubulin RNAs (A), α-tubulin RNAs (B), or rRNAs (C). The resultant autoradiograms are shown. The probe used for the detection of β-tubulin RNAs was constructed from the coding sequences carried by plasmid pT2 (9); probe for α-tubulin was from the coding sequences carried by plasmid pT1 (9); probe for rRNA was from plasmid pXLr101A (the gift of B. Sollner-Webb, Johns Hopkins School of Medicine). This plasmid carries part of the *Xenopus laevis* 18S and 28S ribosomal sequences. The arrow in (A) denotes the position of RNAs derived from the β2 tubulin gene (16).

Secondary chicken fibroblasts were prepared from 10-day White Leghorn embryos and propagated in M119 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 1% heat-inactivated chick serum and 10% fetal calf serum.

S1 analysis of RNA. Unless otherwise indicated, probes used for S1 analysis were generated by 5'-end labeling of calf intestine alkaline phosphatase-treated DNA fragments with polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and [γ-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.). Typically, for the S1 protocol, 5 μg of RNA was coprecipitated with 0.05 pmol of labeled probe and 70 μg of tRNA as the carrier. The pellet was washed with 70% ethanol and dissolved in 20 μl of 80% formamide–0.4 M NaCl–1 mM EDTA–40 mM PIPES [piperazine-*N*-*N'*-bis(2-ethanesulfonic acid)] (pH 6.4) (5). The mixture was heated to 90°C in a tightly sealed tube and left to cool slowly to 60°C. Hybridization was allowed to continue overnight at 60°C. The mixture was then diluted 10-fold and made to 0.25 M NaCl–5 mM $ZnCl_2$–30 mM sodium acetate (pH 4.5). At this point, 100 to 200 U of S1

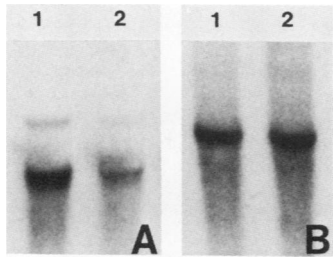

FIG. 2. Modulation of mouse L cell β-tubulin mRNA levels in response to colchicine. RNA was isolated from parallel dishes of L cells, one of which was exposed to 10 μM colchicine for the final 3 h. Equal amounts of RNA from each were analyzed by RNA blotting. (A) The resultant filter was hybridized to the β-tubulin coding sequences from the cDNA plasmid pT2. (B) A duplicate blot was hybridized to 18S ribosomal sequences from the plasmid pXLr101A. In each part of the figure, lane 1 contains RNA from control cells; lane 2 contains RNA from colchicine-treated cells.

nuclease (Bethesda Research Laboratories, Rockville, Md.) and 10 μg of denatured carrier (salmon sperm) DNA were added. The reaction was allowed to proceed at room temperature for 60 min, after which EDTA was added to 10 mM and the nucleic acids were precipitated with ethanol. The labeled, protected probe fragments were sized on 9.0 M urea sequencing gels and visualized by autoradiography.

For the construction of an S1 probe which spanned the transcription initiation site of chicken gene β2, a labeled synthetic oligonucleotide, [^{32}P]-CGGTGTCTTGCCGGTGC, that is complementary to a region of the 5′ untranslated region of β2 RNA (nucleotides 41 to 57 with respect to the cap site) was hybridized to a single-stranded M13 subclone containing the 5′ portion of the β2 gene. The complementary strand that was synthesized with the Klenow fragment of DNA polymerase I and deoxynucleotide triphosphates was used as the probe in S1 mapping analysis.

Blot analysis of RNA. Gels for the separation of RNA were poured from 1% agarose containing 2.2 M formaldehyde (2). RNA was transferred to nitrocellulose (26) and hybridized to ^{32}P-labeled probes as previously described (10). ^{32}P-labeled probes for blot analysis were prepared by the random priming method of Shank et al. (24). The strength of hybridization of ^{32}P-labeled probes to filters was quantitated by scanning autoradiographic tracks with a densitometer (Kontes Co., Vineland, N.J.) equipped with a Hewlett-Packard numeric integrator.

RESULTS

Regulated expression of endogenous chicken and mouse tubulin genes. Virtually all animal cells respond to colchicine-induced microtubule depolymerization with a specific repression (5- to 10-fold) of new tubulin polypeptide synthesis (10). As we have previously shown for cultured CHO cells, this depression is the result of a loss of cellular tubulin RNA sequences (7, 8, 10). That an analogous situation exists for tubulin RNAs in chicken fibroblasts is demonstrated in Fig. 1. Total cellular RNA was prepared from control cells and from cells incubated for 3 or 6 h in 10 μM colchicine. [Although lower concentrations of colchicine ultimately induce microtubule depolymerization, this higher level of drug was utilized to induce complete microtubule depolymerization within 15 min (Pittenger and Cleveland, unpublished data).] Equal amounts of RNA from each sample were then analyzed by RNA blotting. α- and β-tubulin RNAs were detected by hybridization to the cDNA sequences from plasmids pT1 and pT2, respectively (9). Clearly, both α-tubulin (Fig. 1B) and β-tubulin (Fig. 1A) RNAs were depressed ca. fivefold in colchicine-treated cells as compared with control cells. In particular, the 1,800-base RNA, which is derived almost entirely from the chicken β2 gene (16), was down-regulated in response to colchicine treatment. A parallel blot probed for rRNA sequences (Fig. 1C) showed that rRNA signals were comparable in each of the three RNA samples, thus demonstrating that the loss of tubulin RNA sequences in the colchicine sample could not be due to inadvertent differences in RNA quantitation and preparation in control and colchicine-treated cells.

Mouse L cells also respond to colchicine-induced microtubule depolymerization by the specific depression of endogenous tubulin RNAs. As shown in Fig. 2, the exposure of L cells to colchicine for 3 h resulted in a significant loss of β-tubulin RNAs (cf. lanes 1 and 2 in Fig. 2A). Again, this loss was not due to inadvertent differences in RNA quantitation in control and colchicine-treated samples, since a parallel blot probed for 18S ribosomal sequences showed no difference between control and colchicine-derived RNAs (Fig. 2B, lanes 1 and 2).

Taken together, these data indicated that both chicken and mouse cells possess the requisite machinery for tubulin autoregulation. We therefore undertook further experiments to test whether the appropriate regulation of a chicken β-tubulin gene could be reconstituted in the mouse cells after introduction by transfection.

Expression of the chicken β2 gene in mouse L cells. Initially,

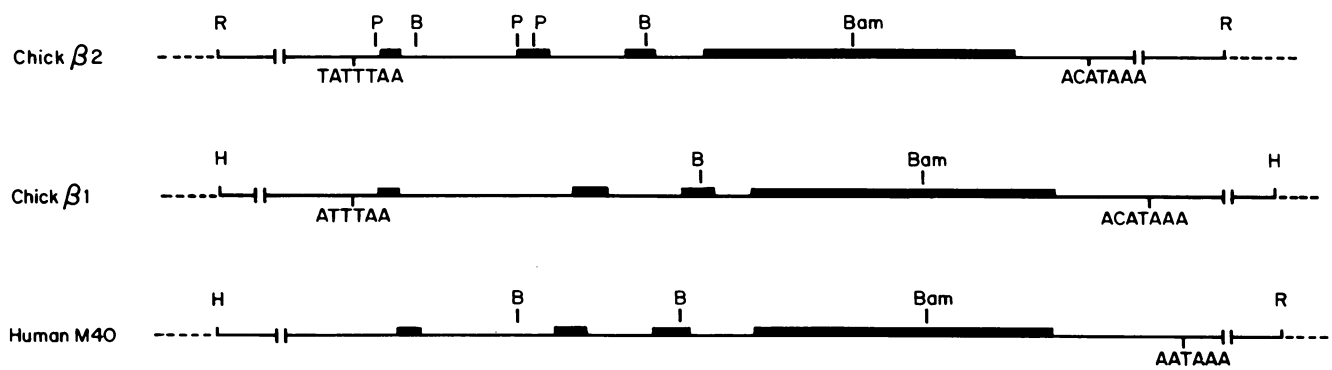

FIG. 3. Schematic diagrams of genes coding for chicken β2 tubulin, chicken β1 tubulin, and human M40 β-tubulin that are contained in pβG2, pβG1, and pM40, respectively. Boxed regions represent exon sequences. Pertinent restriction site abbreviations are as follows: P, *Pst*I; B, *Bam*HI; H, *Hin*dIII; and R, *Eco*RI.

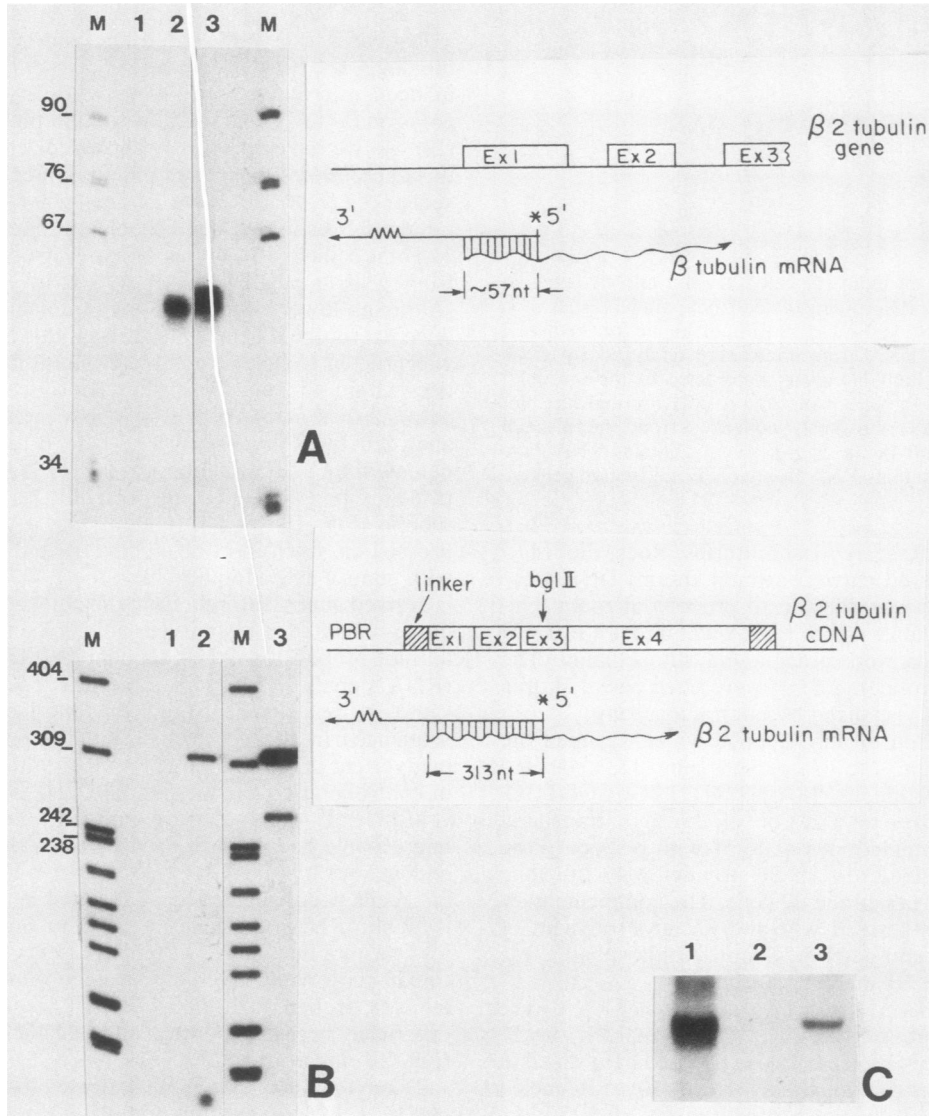

FIG. 4. Synthesis of chicken β2 tubulin mRNA by mouse L cells. RNAs prepared from mock- and pβG2-transfected L cells were analyzed either by S1 mapping analysis (A and B) or RNA blotting (C). For the S1 analysis, equal amounts of RNA recovered from mock- or pβG2-transfected cells were hybridized to a pβG2-specific probe, the mixtures were digested with S1 nuclease, and the S1-resistant fragments were displayed by electrophoresis and autoradiography. In (A), an S1 probe 5′ end labeled at nucleotide 57 of the normal β2 transcript was utilized (details of probe construction are in the text). Lanes 1 to 3, Results obtained with RNAs from mock-transfected cells, pβG2-transfected cells, and chick brain, respectively. In (B), a similar S1 mapping analysis, with the exception that the probe was generated from pT2-H3, a plasmid carrying a cDNA copy of the chicken β2 tubulin gene. This plasmid was opened and 5′ end labeled at the unique *Bgl*II site located 313 nucleotides from start of the cDNA sequence. Lanes 1 to 3, Fragments protected by RNA from mock-transfected cells, pβG2-transfected cells, and chick brain, respectively. M, Molecular weight markers (in nucleotides). (C) An RNA blot analysis with a ^{32}P-labeled probe specific for the 3′ untranslated region of the chicken β2 tubulin mRNA. Lanes 1 to 3, RNA from chick brain, mock-transfected cells, and pβG2-transfected cells, respectively.

to determine whether correct transcription and processing of a transfected chicken β2 tubulin gene could be achieved, we introduced the β2 gene into mouse L cells. Figure 3 is a schematic drawing of the major structural features of this gene, including the four protein-coding exon sequences, the position of the presumptive TATA promoter sequence, and the location of the ACATAAA signal for polyadenylation. The complete nucleotide sequence, starting 275 bases 5′ to the presumptive cap site for RNA transcription through 270 bases 3′ to the site of polyadenylation, has been determined and presented elsewhere (K. F. Sullivan, J. T. Y. Lau, and D. W. Cleveland, submitted for publication). As described previously (21), the entire β2 tubulin gene has been isolated on a 4.5-kilobase *Eco*RI fragment and subcloned into the unique *Eco*RI site of pBR322 in an orientation such that transcription would proceed clockwise in the normal presentation of pBR322.

The ability of L cells to correctly express the β2 tubulin gene and to process its nascent RNA transcript was tested by S1 nuclease analysis. For this, two different end-labeled probes were utilized. To confirm that the expression of the transfected chicken β2 gene was derived from the appropriate β2 transcription initiation site, we used an S1 probe corresponding to sequences spanning the normal transcrip-

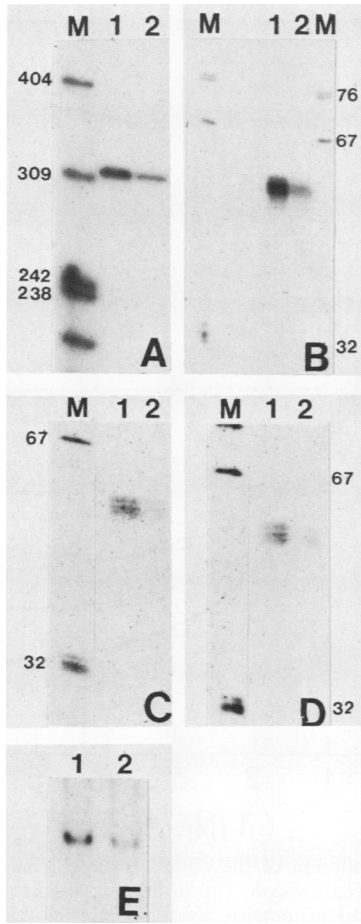

FIG. 5. Effects of colchicine-induced microtubule depolymerization on the level of expression of the chicken β2 tubulin gene introduced by transfection. Duplicate dishes of cells were transfected in parallel with pβG2 DNA. At 23 h posttransfection, colchicine was added to one dish to a final concentration of 10 μM. After 3 additional h, total RNA was prepared from each dish, and equal amounts of RNA from each were assayed for β2-specific RNAs by S1 mapping. In (A), an S1 probe labeled within the third exon sequence (the *Bgl*II probe from pT2-H3 [see the legend to Fig. 4]) was utilized. (B) to (D) each represent analyses of RNAs from independent transfection experiments. In each of these instances, the S1 probe utilized was the one labeled at position 57 of the β2 RNA transcript (as detailed in the text). Lane 1 of each part represents probe protected by RNA from untreated control cells; lane 2 represents probe protected by RNA from colchicine-treated cells. Size markers generated from an *Hpa*II digest of pBR322 were loaded in lanes labeled M, and their appropriate sizes in nucleotides are indicated. (E) RNA blot analysis of the same transfected RNAs which were examined by S1 analysis in (D). Five micrograms of total RNA from control (lane 1) and colchicine-treated (lane 2) cells were blotted and probed for the presence of the transfected β2 RNAs with a ^{32}P-labeled probe specific for the 3′ untranslated region of the chicken β2 tubulin mRNA. An autoradiograph of the resultant hybridization is shown.

tion start site. This probe was 5′ end labeled at a position corresponding to nucleotide 57 of the authentic β2 transcript. The probe was hybridized under R-looping conditions (5) to RNA prepared from L cells which had been transfected with the β2 gene. After digestion with the single-strand-specific nuclease S1, the protected probe fragments were resolved by electrophoresis on DNA sequencing gels and detected by autoradiography. A closely spaced series of protected fragments (57 ± 2 bases, Fig. 4A, lane 2) identical in size to that protected by authentic chicken RNA (Fig. 4A, lane 3) was produced. This S1 probe was specific for transcripts from the chicken gene since endogenous mouse RNAs did not yield any protected fragment(s) (Fig. 4A, lane 1).

To determine whether L cells can appropriately process the chicken tubulin primary transcript, a second S1 probe was prepared from a nearly full-length cDNA clone of the mature β2 mRNA transcript. Plasmid pT2-H3 (which is a subclone of pT2 [9] and contains β2 cDNA sequences starting 61 bases 5′ to the AUG translation initiation codon and terminating at the 3′ polyadenylate tract) was opened at the unique *Bgl*II site which lies 313 nucleotides from the start of the cDNA sequence. After 5′-end labeling, this probe was hybridized to various RNAs and subsequently subjected to digestion with S1. As clearly demonstrated in Fig. 4B, lane 3, authentic chicken mRNA protected the expected 313-nucleotide probe fragment. (The weaker 265-nucleotide signal in lane 4 is derived from the presence of an mRNA encoded by the chicken β1 gene, which diverges in strong sequence homology to β2 265 bases 5′ to the *Bgl*II site [Sullivan et al., submitted for publication].) This S1 probe DNA is, however, specific for the RNA transcripts copied from chicken tubulin genes, since endogenous mouse L cell RNAs do not protect any portion of the probe from S1 digestion (Fig. 4B, lane 1). When RNA prepared from cells 26 h after transfection with the β2 gene was analyzed, the presence of authentic β2 mRNA was demonstrated by the presence of the 313-nucleotide fragment (Fig. 4B, lane 2). Moreover, since the probe was end labeled at a site within the third exon (amino acid position 84), the presence only of a 313-nucleotide protected fragment indicates efficient excision of the first and second introns of the transfected gene transcript.

Finally, to further verify the correct expression of the heterologous chicken mRNA in L cells, RNA from transfected cells was analyzed by RNA blotting with a probe specific for the 3′ untranslated region of chicken β2 mRNA. This probe did not hybridize to endogenous L cell transcripts (Fig. 4C, lane 2). However, a single 1,800-base, β2-specific RNA species was detected in transfected L cells (Fig. 4C, lane 3); moreover, this RNA species was indistinguishable in size from the authentic β2 transcripts present in chicken RNA (Fig. 4C, lane 1).

Regulation of chicken β2 expression in mouse L cells. We next sought to determine whether the level of expression of the chicken β2 gene after introduction into L cells by transient transfection was sensitive to drug-induced microtubule depolymerization as are the endogenous mouse β-tubulin genes (Fig. 2A) or indeed the β2 gene in its normal chromosomal environment (Fig. 1A). To test this, two identical dishes of cells were transfected with the β2 gene, and at 23 h posttransfection colchicine was added to one dish to a final concentration of 10 μM. Three hours later, total RNA was prepared from each dish, and equivalent amounts of RNA from each were examined for the level of β2-specific RNAs by the S1 protocol. The results of that analysis with the probe beginning at the *Bgl*II site within the third exon are shown in Fig. 5A. Remarkably, the level of β2 RNAs in the transfected cells was depressed ca. fivefold as a consequence of treatment with colchicine (cf. lanes 1 and 2). Moreover, to document the reproducibility of the diminution of transfected β-tubulin RNA content after colchicine-induced microtubule depolymerization, the results of three additional, independent transfection experiments are shown

in Fig. 5B to D. For these experiments, the 5′ S1 probe (which spans the transcription initiation site) was utilized to analyze RNA from parallel dishes of transfected cells which either had (lane 2) or had not (lane 1) been treated with colchicine for the final 3 h. Clearly, the transfected β-tubulin RNA declines significantly in all experiments. Furthermore, that the levels of RNA were accurately quantitated by the S1 assay was verified by RNA blot analysis with a labeled probe specific to the transfected β-tubulin RNA (as documented in Fig. 4C). An example of such a blot experiment is shown in Fig. 5E, in which we have reexamined the RNAs from the experiment analyzed by S1 in Fig. 5D. Densitometry of both the S1 (Fig. 5D) and blot analyses (Fig. 5E) confirmed that, in this experiment, the transfected gene RNA levels fell 4.5-fold as measured by either assay.

To determine whether the transfected β2 gene was down-regulated coordinately with the loss of endogenous mouse tubulin gene transcripts, we used blot analysis of RNAs prepared from parallel dishes of transfected cells which had been incubated for increasing lengths of time in colchicine. The hybridization probe specific to the 3′ untranslated region

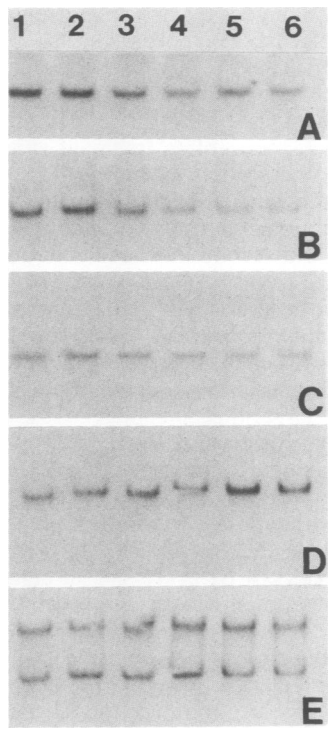

FIG. 6. Effects of colchicine-induced microtubule depolymerization on the level of expression of a transfected chicken β2 tubulin gene and the endogenous mouse tubulin genes as assayed by RNA blotting. Identical dishes of cells that had been transfected with pβG2 were exposed to colchicine for 0, 0.5, 1.5, 3.0, 4.5, and 6.0 h (lanes 1 to 6, respectively). Equal amounts of RNA prepared from these cells were subjected to RNA blot analysis with the following probes. (A) The 3′ untranslated region of chicken β2 mRNA. This probe is specific for the β2 transcript. (B) The β-tubulin cDNA sequences from pT2 (9). This probe recognizes the endogenous mouse β-tubulin RNAs and the transfected chicken β RNAs. (C) The α-tubulin cDNA sequences from pT1 (9). This probe recognizes endogenous mouse α-tubulin RNAs. (D) The β-actin coding sequences from pA1 (9). This probe recognizes endogenous mouse actin RNAs. (E) Plasmid pX1r101A. This probe recognizes mouse rRNAs.

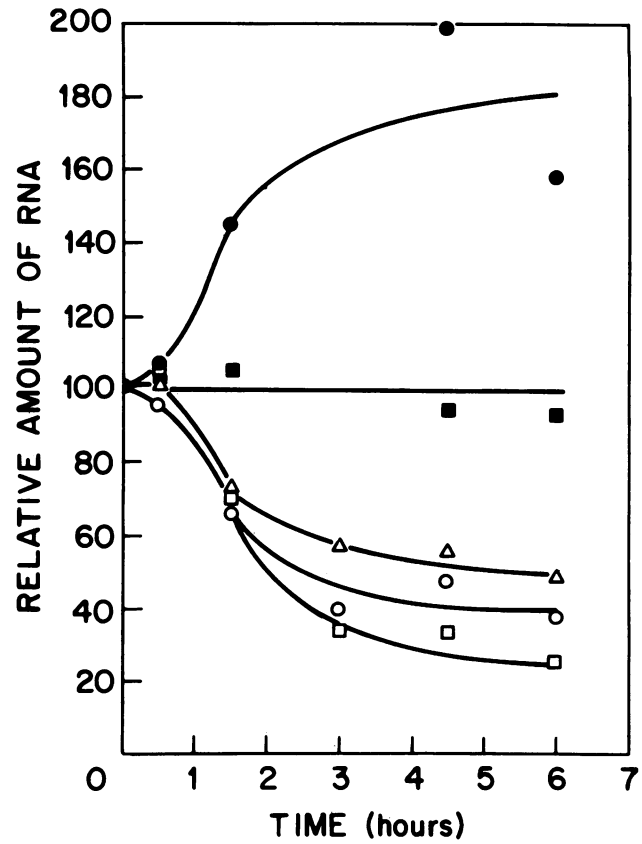

FIG. 7. Quantitation of the effects of colchicine treatment on tubulin and actin expression. The autoradiogram displayed in Fig. 6 was analyzed by densitometric scanning, and the relative levels of each RNA in cells incubated for increasing times in colchicine are shown. Symbols: (●) endogenous actin RNAs; (■) 18S rRNA; (△) endogenous α-tubulin RNAs; (○) transfected chicken β2 RNA; and (□) endogenous β-tubulin RNAs.

of the β2 gene was utilized (Fig. 6A). As expected, in light of the S1 results of Fig. 5, β2-specific RNAs decline in amount upon treatment of the cells with colchicine. A qualitatively similar decline in tubulin RNAs was found when a probe which recognizes total β-tubulin RNAs (both endogenous and transfected gene RNAs) (Fig. 6B) or a probe which hybridizes to endogenous mouse α-tubulin RNAs was utilized (Fig. 6C). This loss of RNA was, however, specific to the tubulins, since actin RNAs were not lost (Fig. 6D). In addition, that the differences in tubulin RNA levels were not due to unintentional loading or blotting artifacts was also examined with a final blot which was probed for rRNA sequences (Fig. 6E). No differences in rRNA amount were apparent.

To quantitate the various RNA levels as a function of time of incubation in colchicine, we analyzed the autoradiograms displayed in Fig. 6 by densitometry. The results of this quantitation are plotted in Fig. 7. As is obvious by inspection of the figure, rRNA levels were in fact constant, whereas tubulin RNAs were depressed by a factor of ca. 3 during the time course. Moreover, the transfected chicken β2 transcripts were lost at a rate indistinguishable from the rate of loss of the endogenous α- and β-tubulin sequences. Curiously, actin RNA levels were affected in the opposite direction, showing a ca. twofold increase. This response, perhaps due to an indirect effect of colchicine on the actin

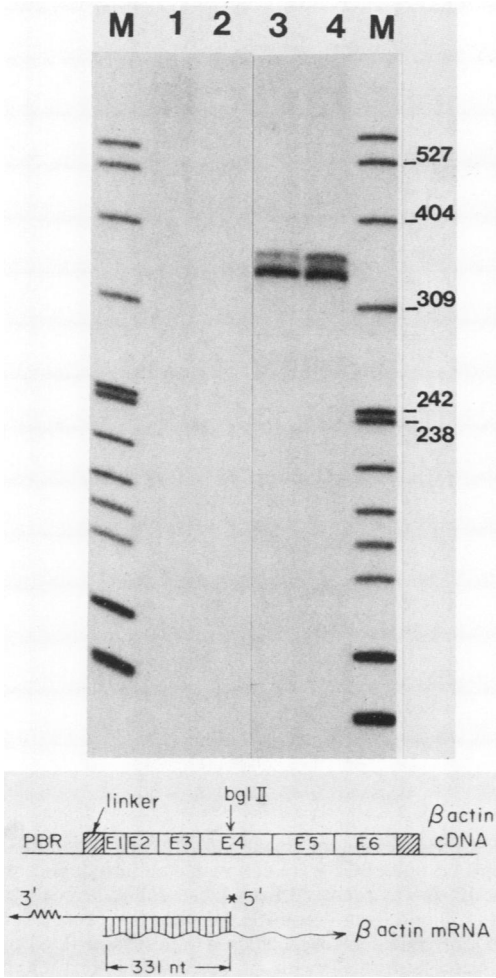

FIG. 8. Regulated expression of chicken β-actin after transfection into mouse L cells. Multiple dishes of L cells were mock transfected or transfected with the plasmid pβAct, which contains the complete chicken β-actin gene. Three hours before harvesting, colchicine was added to some dishes to a final concentration of 10 μM. Total RNA was prepared from each dish, and equal amounts were analyzed by S1 mapping for the presence of chicken β-actin RNA transcripts. An appropriate S1 probe (derived from plasmid pA1 [9], which carries a nearly full-length copy of chicken β-actin RNA) was opened and end labeled at the unique *Bgl*II site located in the fourth exon of the β-actin sequence. Lanes 1 and 2, Probe fragments protected by mock-transfected cells; lanes 3 and 4, corresponding fragments from pβAct-transfected cells. Lanes 2 and 4, cells treated with colchicine; lanes 1 and 3, cells not treated with the drug. *Hpa*II-digested pBR322 fragments were used as size markers (lanes M), and their appropriate sizes in nucleotides are as indicated.

network, is quite reproducible. Moreover, as we document below, the expression of a heterologous, transfected β-actin gene is similarly elevated by colchicine.

Antimicrotubule drug-induced down-regulation is specific to transfected tubulin gene sequences. The obvious implication of the transfection data presented in Fig. 5 and 6 is that L cells are able to recognize and appropriately regulate the expression of an unintegrated, heterologous β-tubulin gene. Presumably, one or more regions of DNA sequence located on the β2 transcription unit contain a necessary regulatory region(s). However, these data alone do not exclude the possibility that the observed down-regulation in response to colchicine treatment is the result of a nonspecific response arising as a consequence of nontubulin sequences present on the plasmid vector or, alternatively, as an artifact of the transfection protocol.

To test this possibility, we have examined the effects of colchicine on the expression of a plasmid carrying the chicken β-actin gene. The actual plasmid, pβAct (kindly provided by T. Kost and S. Hughes [18]), contains the entire chicken β-actin gene cloned on an 8.9-kilobase fragment inserted in a clockwise orientation into the *Eco*RI site of pBR322. This plasmid was either introduced into L cells alone or cotransfected along with the β2 tubulin plasmid. At 26 h posttransfection, some dishes of cells were treated with 10 μM colchicine; 3 h later, RNA was prepared in parallel from control and drug-treated cells and subjected to S1 analysis for the level of expression of both chicken β-actin RNA and chicken β2-tubulin RNA.

Figure 8 shows the results of transfection of the β-actin gene alone. The probe utilized for analysis of the expression of the chicken actin gene was isolated by restriction cleavage of a nearly full-length chicken β-actin cDNA clone (clone pA1 [9]) at the *Bgl*II site which lies 331 nucleotides from the 5′ end of the cDNA. Hence, after 5′-end labeling, hybridization to authentic chicken β-actin RNA should yield a 331-base S1-protected fragment. Such a fragment was in fact observed with RNA derived from cells transfected with plasmid pβAct (Fig. 8, lane 3). Endogenous mouse actin RNAs did not, however, protect any portion of the probe from digestion (Fig. 8, lanes 1 and 2). When RNA was analyzed from cells which had been transfected with pβAct and subsequently incubated in colchicine for 3 h, an increase in transfected actin RNAs was observed (Fig. 8, lane 4). Clearly, unlike the situation found for the β2 tubulin gene, colchicine treatment of cells transfected with an actin gene does not result in depression of the level of actin expression.

This finding is established even more firmly in cells cotransfected with both the β2 tubulin and β-actin genes. RNA isolated from parallel dishes of transfected cells which had been incubated for the final 3 h in the absence or presence of colchicine was analyzed by the S1 technique for the expression of both genes. As is evident in Fig. 9, lanes 3 and 4, β-tubulin RNAs declined by ca. threefold as the result of colchicine treatment, whereas β-actin RNAs actually increased by ca. twofold (Fig. 9, lanes 1 and 2). As noted above, this increase in actin RNAs parallels that found for the endogenous actins after treatment with colchicine.

Regulation of other vertebrate β-tubulin genes after transfection. To determine whether the appropriate down-regulation after drug-induced microtubule depolymerization was unique to the chicken β2 tubulin gene or whether such regulation was a general feature of transfected vertebrate β-tubulins, we have analyzed the expression of two additional β-tubulin genes after transient transfection into L cells. The first of these is the chicken β1 gene whose structure is shown in Fig. 3. Although this gene is extraordinarily homologous to the β2 gene in nucleotide sequences in the protein-coding regions, it diverges in nucleotide sequences in the promoter, the 5′ untranslated region, the first intron, and the 3′ untranslated region sequences (Sullivan et al., submitted for publication). To assay for the expression of this gene after transfection, we utilized the S1 probe beginning at the *Bgl*II site within the cDNA of pT2. As shown earlier (Fig. 4B, lane 3), although this probe is actually a cDNA copy of the RNA transcript from the β2 gene, sufficient homology is present in the authentic chicken RNA transcript from the β1 gene to protect a probe fragment

of 265 nucleotides in length. (This 265-base-pair fragment corresponds to the known region of sequence homology between β1 and β2 transcripts, extending from 8 nucleotides 5′ to the AUG translation initiation codon through the *Bgl*II site at amino acid residue position 84.) When this probe was used to analyze RNA from cells transfected with the β1 gene, the expected 265-nucleotide protected fragment was detected (Fig. 10A, lane 1), indicating that the β1 gene was transcribed and most probably properly processed. When the level of the β1 tubulin RNA in control and colchicine-treated transfected cells was compared (cf. lanes 1 and 2 of Fig. 10A), it was apparent that the β1 transcripts were depressed ca. twofold in the colchicine-treated cells. Hence, this gene is down-regulated in a qualitatively similar fashion to that already reported for β2 tubulin.

Finally, we have investigated the expression of the human β-tubulin gene M40 (19). The important structural features of this gene are shown in Fig. 3. This gene (generously provided by N. Cowan) was subcloned in a counterclockwise orientation between the *Hin*dIII and *Eco*RI sites of pBR322. For S1 analysis, a probe (prepared from the M40 gene itself) was 5′ end labeled at the unique *Bam*HI site which lies 86 bases 3′ to the third intron-fourth exon junction. Thus, when this probe is utilized to analyze mature processed RNAs, a protected fragment of 86 bases would be expected. After transfection of the M40 gene into L cells, the expression of the transfected gene was confirmed by the presence of this 86-base protected fragment (Fig. 10B, lane 2). Under our S1 conditons, however, the probe was not completely specific to the human gene since a very weak S1 signal was detected in mock-transfected cells (Fig. 10B, lane 1). Nonetheless, mindful of this minor contaminating signal, it was still clear in comparing the level of M40 RNA transcripts in control (Fig. 10B, lane 2) and colchicine-treated (Fig. 10B, lane 3) transfected cells that colchicine produced a marked (ca. fivefold) depression in the level of M40 RNAs.

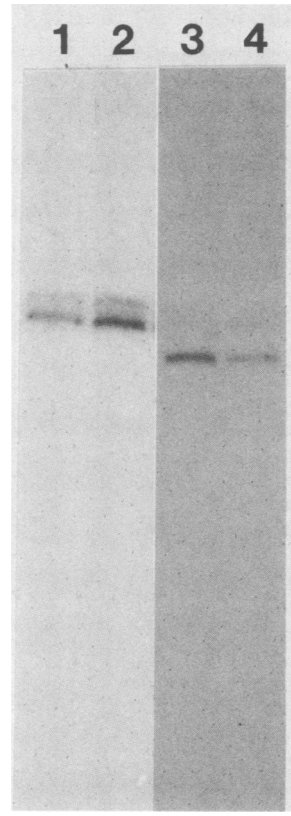

FIG. 9. Cotransfection of chicken β-actin and β2 tubulin genes. Plasmids pβAct and pβG2 were cotransfected into duplicate dishes of L cells. RNA was prepared from these doubly transfected cells (lanes 1 and 3) and from cotransfected cells that were exposed to colchicine for the final 3 h of incubation (lanes 2 and 4). S1 mapping analyses were performed with probes specific for the chicken β-actin mRNA (lanes 1 and 2) or the chicken β2 tubulin mRNA (lanes 3 and 4). The construction of each of these probes is described in the legends to Fig. 4 and 8.

DISCUSSION

Our present data have demonstrated that cultured L cells efficiently express heterologous tubulin RNAs after transient transfection with appropriate genes carried on plasmid vectors. The heterologous tubulin RNA transcripts are, at least for the chicken β2 tubulin gene, processed to yield correctly spliced mature mRNAs which are identical in size to the authentic chicken RNAs. In view of the many eucaryotic genes which have been successfully expressed by this kind of methodology, this finding is not particularly unexpected. More remarkable is that the expression of the heterologous tubulin genes is subject to the same pattern of control as that of the endogenous tubulin genes. Using colchicine to induce rapid depolymerization of microtubule networks and to raise the intracellular concentration of free tubulin subunits, we have found that the levels of RNAs encoded by the endogenous mouse tubulin genes and the exogenously introduced transfected genes are coordinately down-regulated. Such regulation has been found for each of three β-tubulin genes examined. This repressibility concomitant with microtubule depolymerization is, however, specific to tubulin genes, as indicated by the failure of an actin gene to display similar regulation.

The sum of these data mandates the conclusion that the requisite recognition signal(s) for appropriate tubulin gene regulation by the apparent pool size of depolymerized tubulin subunits must reside directly in the primary tubulin gene DNA sequences. Furthermore, since appropriate modulation was achieved on heterologous tubulin gene sequences that were presumably present as unintegrated, episomal copies, chromosomal position effects cannot constitute an important factor in this regulatory pathway. Similarly, it is equally unlikely that a specific chromatin structure is required for tubulin autoregulation, since this structure would have to form on the naked tubulin gene DNA which is flanked not by the normal eucaryotic regions but rather by plasmid sequences. Finally, since the plasmid DNAs utilized for transfection were prepared by growth in bacteria, requisite regulatory signals cannot reside in specific modifications to particular nucleotides (such as methylation, etc.) unless such events occur on the plasmid-bound genes after transfection.

An unexpected finding emerging from the present work is that actin RNA levels in L cells are elevated after colchicine-induced microtubule depolymerization. However, since microtubules act in concert with actin filaments and intermediate filaments to establish the internal cytoarchitecture, it seems reasonable that perturbations of the cytoskeleton by antimicrotubule drugs might directly or indirectly influence actin expression as well. Furthermore, in response to microtubule depolymerization, a transfected β-actin gene shows an elevated level of expression which parallels that displayed by the endogenous actin genes. Thus, as we have already seen in the preceding tubulin example, the requisite

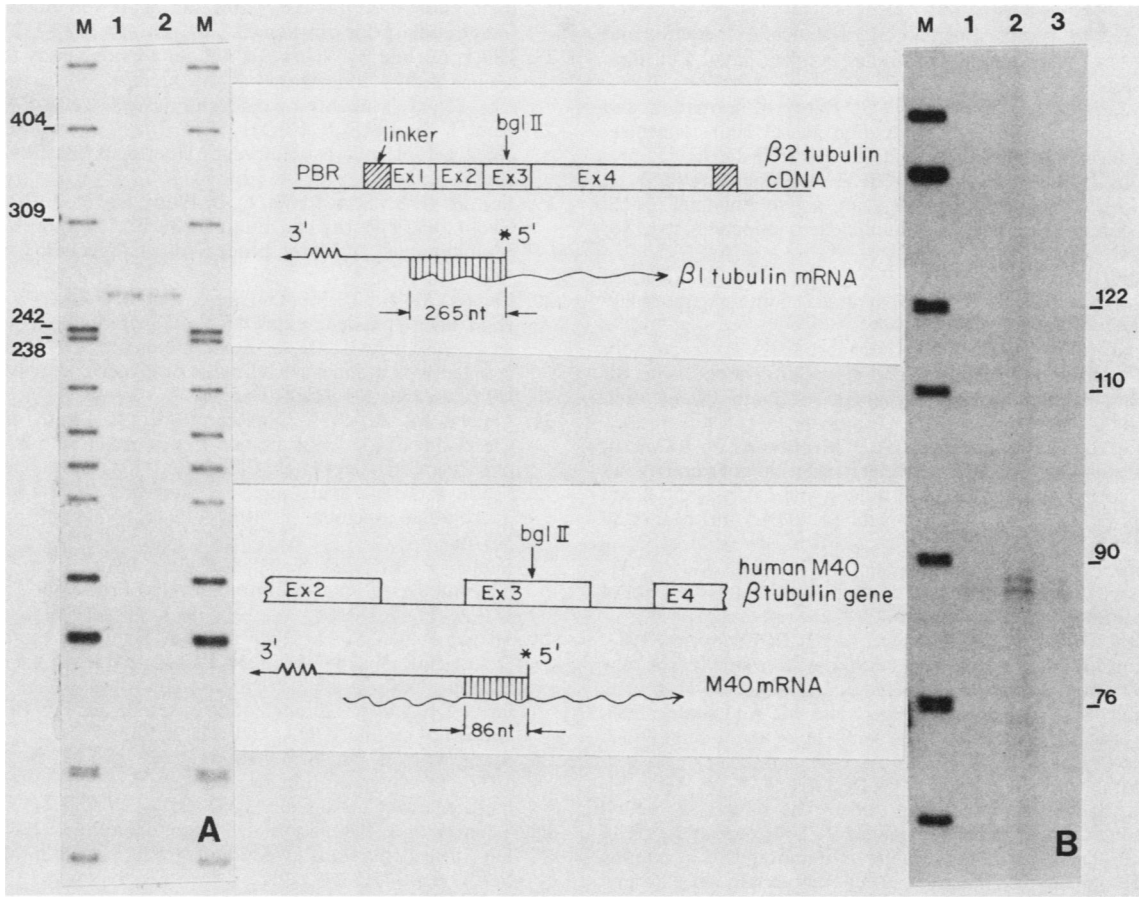

FIG. 10. Regulated expression of chicken β1 tubulin and human M40 β-tubulin RNAs in L cells. (A) pβG1, a plasmid containing the complete chicken β1 tubulin gene, was transfected into duplicate dishes of L cells. S1 analysis was performed on equal amounts of RNA prepared from control cells (lane 1) and from cells incubated for the final 3 h in colchicine (lane 2). The probe utilized was generated from the *Bgl*II site within the cDNA sequence of pT2-H3 (see the legend to Fig. 4). (B) pM40, a plasmid containing the complete human M40 β-tubulin gene, was transfected into L cells. A probe generated by 5'-end labeling of the unique *Bgl*II site in the third exon of the M40 β-tubulin gene sequence was used. The results obtained with equal amounts of RNA obtained from mock-transfected cells (lane 1), pM40-transfected cells (lane 2), and pM40-transfected cells that were exposed to colchicine for 3 h (lane 3) are shown. Size markers generated from *Hpa*II-digested pBR322 are shown (lanes M), and their appropriate sizes in nucleotides indicated.

regulatory sequences must also reside in the primary actin nucleic acid sequences.

Finally, previous work measuring apparent tubulin transcription rates in nuclei isolated from control or colchicine-treated cells has strongly suggested that the loss of cellular tubulin RNA sequences in response to microtubule depolymerization is not the result of down-regulation of tubulin gene transcription (7). If this is in fact correct, then modulation must be achieved either by alterations in the rates or efficiencies of processing or transport of tubulin RNAs in the nucleus or by changes in the rate of cytoplasmic tubulin RNA degradation. The determination of the precise molecular events involved is certainly a technically challenging problem. The demonstration that proper tubulin gene regulation can be achieved on a cloned tubulin gene that has been reintroduced into cultured cells affords a powerful and potentially tractable tool for further investigation of the DNA sequences required to specify regulated tubulin expression. As a consequence, it seems very likely that by construction and analysis of hybrid tubulin genes containing tubulin promoter, coding, intron, or flanking region sequences, the requisite regulatory sequences and pathway of regulation can be identified.

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