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Stabilization of Tubulin mRNA by Inhibition of Protein Synthesis in Sea Urchin Embryos

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An increased level of unpolymerized tubulin caused by depolymerization of microtubules in sea urchin larvae resulted in a rapid loss of tubulin mRNA, which was prevented by nearly complete inhibition of protein synthesis. Results of an RNA run-on assay indicated that inhibition of protein synthesis does not alter tubulin gene transcription. Analysis of the decay of tubulin mRNA in embryos in which RNA synthesis was inhibited by actinomycin D indicated that inhibition of protein synthesis prevents the destabilization of tubulin mRNA. The effect was similar whether mRNA was maintained on polysomes in the presence of emetine or anisomycin or displaced from the polysomes in the presence of puromycin or pactamycin; thus, the stabilization of tubulin mRNA is not dependent on the state of the polysomes after inhibition of protein synthesis. Even after tubulin mRNA declined to a low level after depolymerization of microtubules, it could be rescued by treatment of embryos with inhibitors of protein synthesis. Tubulin mRNA could be induced to accumulate prematurely in gastrulae but not in plutei if protein synthesis was inhibited, an observation that is indicative of the importance of the autogenous regulation of tubulin mRNA stability during embryogenesis. Possible explanations for the role of protein synthesis in the control of mRNA stability are discussed.

In cultured mammalian cells the rate of tubulin synthesis is dependent on the level of unpolymerized tubulin. An increased level of unpolymerized tubulin caused by depolymerization of microtubules in cells treated with colchicine results in a rapid decline in tubulin synthesis (6, 13). This decline is largely the result of a rapid decline in the concentration of tubulin mRNA (10, 13), but the transcriptional activity of tubulin genes, based on a run-on assay in isolated nuclei, is unaltered in cells treated with colchicine (12). The rapid loss of tubulin mRNA following depolymerization of microtubules occurs in enucleate cytoplasts, indicating that the mRNA is destabilized (11, 34).

A similar relationship between the stability of tubulin mRNA and the level of unpolymerized tubulin has been observed in sea urchin embryos following hatching; this observation was based on the use of antimicrotubule agents (21). In addition, the developmental profile of the sensitivity of tubulin synthesis to these agents, as well as the ontogenetic increase in the stability of tubulin RNA, indicates that the increasing polymerization of maternal stores of tubulins during embryogenesis accounts for an increasing rate of tubulin synthesis during later embryogenesis via an autogenous regulatory mechanism similar to that operating in cultured mammalian cells (21; Z. Gong and B. P. Brandhorst, Mol. Repro. Dev., in press).

We have proposed that autogenous regulation of protein synthesis at the level of mRNA stability may be an important regulatory mechanism for the expression of various genes during embryogenesis; a cotranslational mechanism has been proposed for this autogenous regulation (8, 9). Nascent polypeptides would compete with unassembled subunits of oligomeric and polymeric structures for limiting binding sites, possibly on the cytomatrix; if such binding were to result in an increased stability of mRNA in bound polysomes compared with that in unbound polysomes, an autogenous regulatory cycle would be established. The model requires no direct interactions between an mRNA and its translation product and is based on protein-protein interactions that are likely to occur for proteins such as tubulin. Consistent with this model is the observation that a 49-nucleotide sequence encoding the amino terminus of the protein is sufficient for the autogenous regulation of β-tubulin mRNA in mammalian cells (19).

A prediction of this model is that inhibitors of protein synthesis which impede peptide chain elongation, resulting in stabilization of the polysomes, might maintain or increase the stability of the tubulin mRNAs in cells treated with microtubule-depolymerizing agents, while agents which inhibit initiation or disrupt the mRNA-ribosome-nascent peptide complex might destabilize tubulin mRNA even when the level of unpolymerized tubulin is low. We thus treated sea urchin embryos with various inhibitors of protein synthesis and observed the effects on the prevalence and stability of tubulin mRNA. Inhibitors which either stabilize or dissociate polysomes caused the stabilization of tubulin RNA in embryos treated with colcemid and an ontogenetically precocious accumulation of tubulin mRNA. Thus, continued protein synthesis or translation of tubulin mRNA is required for the destabilization of tubulin mRNA by elevated levels of unpolymerized tubulin.

MATERIALS AND METHODS

Colcemid (demecolchicine), vinblastine, nocodazole {methyl[5-(2-thienylcarbinyl)-1*H*-benzimidazole-2-yl]carbonate}, actinomycin D, anisomycin, puromycin, and emetine were purchased from the Sigma Chemical Co. (St. Louis, Mo.); pactamycin was kindly provided by The Upjohn Co. (Kalamazoo, Mich.). The two tubulin cDNA plasmids used, $p\alpha_2$ and $p\beta_2$, were the gift of Joan Ruderman; they included 3′-noncoding and partial coding sequences of α- and β-tubulins, respectively (1). Subcloning of tubulin coding sequences from $p\alpha_2$ and $p\beta_2$ into Bluescript vector (Stratagene) to yield plasmids $b\alpha_2T3c$ and $b\beta_2T7c$ was done as described previously (Z. Gong and B. P. Brandhorst, submitted for publication). Single-stranded RNA complementary to partial α- and β-tubulin-coding sequences were

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prepared by using T3 and T7 polymerases (Bethesda Research Laboratories, Gaithersburg, Md.), respectively.

Lytechinus pictus sea urchins were purchased from Marinus (Long Beach, Calif.). Gametes were obtained and cultures were established as described by Klein et al. (30). Embryos were cultured in artificial seawater (reagent grade) at 18°C with stirring at 60 rpm. Under these conditions, hatching usually occurred at about 12 h after insemination, gastrulation began at 20 h, prisms were formed by 48 h, and early plutei were formed by 60 h. For testing the effect of drugs on protein or RNA synthesis, [^{35}S]methionine was added usually at 1 μCi/ml and [^{3}H]uridine was added at 2 μCi/ml. Labeling was done for 0.5 h (or as specified throughout the text), and radioactive incorporation was determined by precipitation with trichloroacetic acid. Preparation of total cytoplasmic RNA by precipitation with LiCl from a buffer containing urea and RNA gel blot analysis were carried out as described previously (20).

Cytoplasmic extracts were prepared and polysomes were analyzed essentially as described previously (5). The 15,000 × *g* supernatant was layered over a linear 15 to 55% (wt/vol) sucrose gradient and centrifuged at 32,000 rpm for 150 min in a rotor (SW41; Beckman Instruments, Inc., Fullerton, Calif.). The optical density at 254 μm was recorded, and fractions were taken for the preparation of RNA for gel blot analysis, as described previously (36).

The levels of soluble tubulin were analyzed by extraction of embryos under conditions which stabilize microtubules (Gong and Brandhorst, in press). Tubulin was immunoprecipitated from the extracts with an excess of anti-tubulin antibody and analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, as described previously (20).

Nuclei were prepared by a modification of the method of Morris and Marzluff (32), as described by Conlon et al. (14). RNA chain extension was done by the method of Conlon et al. (14) in the presence of [α-^{32}P]UTP. Under these conditions there was strand-specific transcription of some genes. Run-on RNA was prepared from nuclei, essentially as described by Groudine et al. (25).

Quantification of autoradiograms was by densitometry by use of a scanning densitometer (1650; Bio-Rad Laboratories, Richmond, Calif.) connected to an integrator (3390A; Hewlett-Packard Co., Palo Alto, Calif.). Multiple exposures were made to ensure linearity. Gels and blots were stained with ethidium bromide to ensure that essentially the same amounts of RNA were present in the samples that were compared (except for fractions of the polysome profile), corresponding to equal numbers of embryos; this was confirmed in some instances by hybridization to a nick-translated cloned cDNA probe specific for rRNA.

RESULTS

Effects on embryos of inhibitors of protein and RNA synthesis. Four inhibitors of protein synthesis were employed in this investigation: anisomycin, emetine, pactamycin, and puromycin. The effects of these inhibitors on protein synthesis in gastrula embryos were tested by monitoring the incorporation of 30-min pulses of [^{35}S]methionine into protein. All four agents inhibited incorporation by more than 90% at the concentrations of inhibitors normally used in the course of these investigations: 25 μM anisomycin, 30 to 50 μM emetine, 2.5 to 4 μg (4.5 to 7.2 μM) of pactamycin per ml, and 100 to 150 μM puromycin (Fig. 1A). The extent of inhibition of incorporation by these agents was somewhat

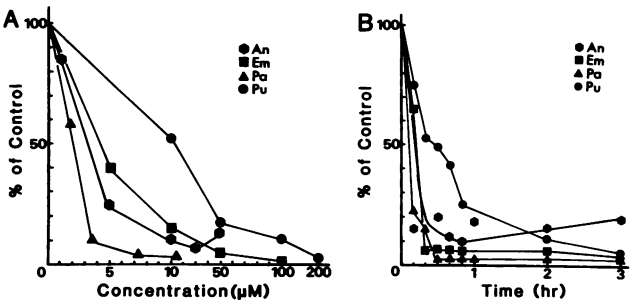

FIG. 1. Inhibition of protein synthesis by inhibitors of protein synthesis. (A) Equal numbers of gastrulae were treated with anisomycin (An), emetine (Em), pactamycin (Pa), or puromycin (Pu) at various concentrations for 3 h and incubated with equal amounts of [^{35}S]methionine for 30 min; and acid-precipitable incorporation was monitored by scintillation counting. The levels of radioactive incorporation relative to that of an untreated control (100%) are plotted. (B) Gastrulae were treated with 30 μM anisomycin (An), 50 μM emetine (Em), 4 μg of pactamycin (Pa) per ml, or 100 μM puromycin (Pu). Equal numbers of untreated control embryos and inhibitor-treated embryos were collected at various times following initiation of inhibitor treatment and were incubated with [^{35}S]methionine for 10 min. The levels of radioactive incorporation in treated embryos relative to that in the corresponding control embryos are plotted. The times indicate the midpoint of the labelling period.

variable among batches of embryos, and prolonged (more than 6 h) treatment of embryos with pactamycin and emetine was sometimes lethal. Treatment of embryos with pactamycin resulted in a thickening of the ectodermal cells of the animal half. The time courses of inhibition of amino acid incorporation by these agents are shown in Fig. 1B. The rate of protein synthesis declined more slowly with puromycin, which was the only inhibitor used that had a reversible effect on methionine incorporation (data not shown).

The stability of a transcript can be estimated from the kinetics of its decay after inhibition of RNA synthesis; actinomycin D was used in these investigations. The effects of various concentrations of actinomycin D on incorporation of a 20-min pulse of [^{3}H]uridine are shown in Fig. 2A. The time course of inhibition of incorporation of a pulse of uridine by 70 μg of actinomycin D per ml is shown in Fig. 2B; by 3 h incorporation was reduced by over 90%. Treatment of embryos with 70 μg of actinomycin D per ml did not

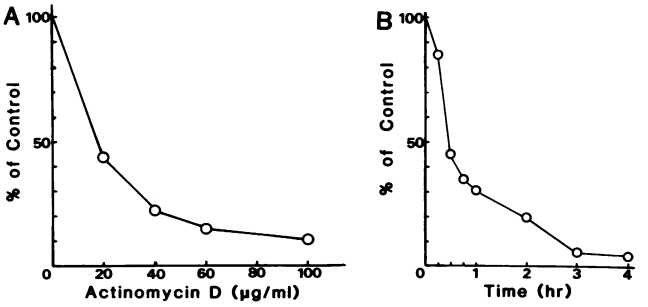

FIG. 2. Inhibition of RNA synthesis by actinomycin D. (A) Equal numbers of gastrulae were treated with different concentrations of actinomycin D for 3 h, labeled with equal amounts of [^{3}H]uridine for 20 min, analyzed, and plotted as described in the legend to Fig. 1A. (B) Gastrulae were treated with 70 μg of actinomycin D per ml, analyzed, and plotted as described in the legend to Fig. 1B, except that the labeling was with [^{3}H]uridine and was for 15 min.

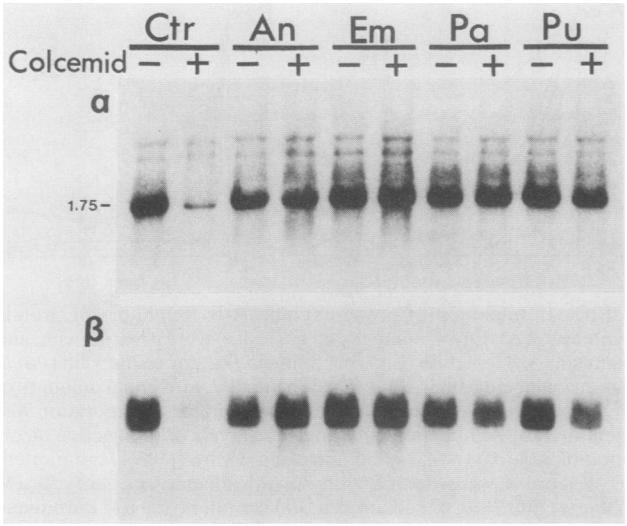

FIG. 3. Prevention of the loss of tubulin mRNAs in colcemid-treated embryos by inhibition of protein synthesis. Early plutei were treated with 30 μM anisomycin (An), 50 μM emetine (Em), 4 μg of pactamycin (Pa) per ml, or 100 μM puromycin (Pu) in the presence (+) or absence (−) of 5 μM colcemid. Inhibitors of protein synthesis and colcemid were added at the same time. Cytoplasmic RNA was isolated 4 h after the initiation of treatment, separated by electrophoresis through agarose gels, blotted onto GeneScreen, and hybridized to synthetic RNA-coding sequence probes transcribed from $b\alpha_2T3c$ (α) or $b\beta_2T7c$ (β). Equal amounts (5 μg) of RNA were applied to each lane. The major α-tubulin transcript (1.75 kilobases) is indicated. Ctr, Control embryos without inhibition of protein synthesis.

result in obvious morphological abnormalities during the experimental period (usually 6 h or less).

Inhibition of protein synthesis prevents the decline in tubulin mRNA in embryos treated with microtubule-depolymerizing agents. Early pluteus embryos were treated with colcemid for 4 h, and cytoplasmic RNA was prepared. RNA gel blot hybridization analysis confirmed previous observations (21) that colcemid treatment results in a substantial decline in the prevalence of tubulin RNA (Fig. 3). This loss of tubulin mRNA was largely or entirely prevented by the concurrent application of any of the four inhibitors of protein synthesis, anisomycin, emetine, pactamycin, or puromycin, at concentrations which inhibited methionine incorporation by more than 90%. Moreover, the application of these inhibitors of protein synthesis had no major effect on the prevalence of tubulin mRNA in plutei that were not treated with colcemid. Thus, in the absence of protein synthesis, release of mRNA from polysomes did not result in the destabilization of tubulin mRNA.

Protection from the loss of tubulin transcripts in the presence of colcemid requires a substantial inhibition of protein synthesis. Treatment of colcemid-treated embryos with 10 μM puromycin, which resulted in less than 50% reduction in protein synthesis, did not prevent the decline in tubulin mRNA (data not shown). Moreover, treatment of embryos with cycloheximide, which inhibited amino acid incorporation by only 50%, did not prevent the decline of tubulin mRNA resulting from colcemid treatment (data not shown).

Inhibition of protein synthesis stabilizes tubulin mRNA in embryos treated with colcemid. The prevention by inhibitors of protein synthesis of the loss of tubulin mRNA in embryos treated with microtubule-depolymerizing agents was

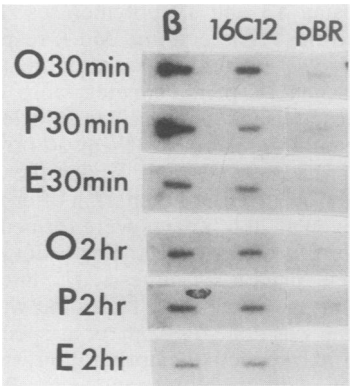

FIG. 4. Run-on assay of transcriptional activity of β-tubulin genes in the presence of inhibitors of protein synthesis. Gastrula embryos were treated with 100 μM puromycin (P) or 50 μM emetine (E). Nuclei were isolated 30 min or 2 h after the initiation of treatment and incubated with [^{32}P]UTP under conditions that allowed RNA chain extension. RNA was extracted from the nuclei, and run-on-labeled RNA samples (equal amounts of radioactivity) were hybridized to a set of plasmid DNAs (5 μg in each slot) blotted onto GeneScreen. Lanes: β, $p\beta_2$, a β-tubulin DNA; 16C12, a cDNA clone from an *L. pictus* gastrula cDNA library (14); pBR, pBR322, the vector for $p\beta_2$ and 16C12. O, Untreated control embryos.

achieved by either an increase in tubulin mRNA synthesis or an increase in tubulin mRNA stability. The effects of the inhibitors of protein synthesis on transcription of tubulin genes were examined by using an RNA run-on assay. Gastrula embryos were treated with puromycin or emetine for 30 min or 2 h, and nuclei were prepared and incubated with [^{32}P]UTP under conditions that allowed RNA chain extension; run-on-labeled RNA was hybridized to excess β-tubulin DNA. No increase in the relative transcriptional activity of tubulin genes was observed (Fig. 4); in fact, the relative transcriptional activity of the β-tubulin gene and another unidentified gene (16C12) appeared to be somewhat depressed by the inhibition of protein synthesis. Thus, protection from the loss of tubulin mRNA by inhibitors of protein synthesis appeared to be posttranscriptionally mediated.

The kinetics of decay of cytoplasmic tubulin RNA was monitored after the inhibition of RNA synthesis with actinomycin D (Fig. 5). As reported previously (21), colcemid treatment of early pluteus embryos results in the destabilization of β-tubulin mRNA (as well as α-tubulin RNA). This destabilization was largely prevented by inhibition of protein synthesis with puromycin. Treatment with puromycin alone appeared to result in a slight destabilization of β-tubulin RNA, while treatment with emetine resulted in an increased stability of β-tubulin RNA. Similar results were obtained for α-tubulin RNA and are summarized in Table 1. Results of these and other similar experiments indicate that inhibitors of protein synthesis protect tubulin mRNA from the destabilization caused by an elevated level of unpolymerized tubulin.

These observations indicate that inhibition of protein synthesis, or the prevention of translation of the tubulin mRNA, prevents the destabilization of tubulin mRNA resulting from colcemid treatment. This conclusion is based on the well-established effects of these protein synthesis inhibitors. The stabilization of polysomes in sea urchin embryos by treatment with the standard concentration of emetine has been demonstrated previously (27, 28), as has the dissociation of polysomes by pactamycin at standard concentrations

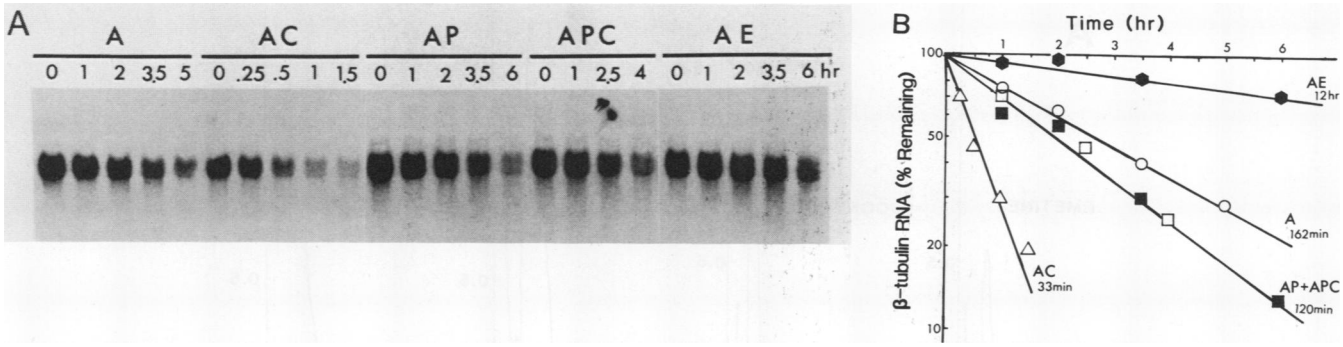

FIG. 5. Decay of β-tubulin transcripts in embryos treated with colcemid, inhibitors of protein synthesis, or both in the presence of actinomycin D. (A) Early plutei were treated with 70 μg of actinomycin D per ml (A), 70 μg of actinomycin D per ml–100 μM puromycin (AP), or 70 μg of actinomycin D per ml–50 μM emetine (AE). Colcemid (5 μM) was added to portions of treatment groups A and AP 1 h later to form groups AC and APC, respectively. Samples of embryos were taken beginning at the time of colcemid addition (time zero, which was 1 h after the initiation of treatment with actinomycin D and protein synthesis inhibitors). Cytoplasmic RNA (5 μg per lane) was analyzed by RNA gel blot hybridization, using nick-translated $p\beta_2$ DNA as a probe. (B) The autoradiographs shown in panel A were quantified by densitometry, and the concentrations of β-tubulin RNA relative to time zero (100%) are plotted against time on a semilogarithmic scale for various times after the addition of colcemid. Half-lives estimated from linear fits of the decay data are shown for each curve. Symbols: ○, control embryos (A); △, colcemid-treated embryos (AC); hexagon; emetine-treated embryos (AE); □, colcemid-treated embryos in the presence of puromycin (APC); ■, puromycin-treated embryos (AP).

(17). To eliminate the possibility that colcemid treatment alters the action of these protein synthesis inhibitors, we analyzed the polysome profiles of embryos that were treated simultaneously with colcemid and one of these inhibitors. The fraction of ribosomes in polysomes and the size distribution of polysomes were increased in embryos treated with emetine, while pactamycin and puromycin treatment effectively dissociated polysomes (Fig. 6). Polysomes were maintained in embryos that were treated with anisomycin but at a level similar to that for control embryos, which is consistent with the reduced effectiveness of this inhibitor (data not shown). While polysomes were stabilized by emetine treatment, it is possible that tubulin mRNA might be selectively released by colcemid treatment. RNA gel blot analysis of RNA extracted from fractions across the polysome profile is shown in Fig. 6B. In embryos treated with emetine and colcemid, as well as embryos treated with colcemid (or not treated), β-tubulin transcripts were almost entirely restricted to the polysome fraction. Thus, the stabilization of tubulin mRNA by inhibitors of protein synthesis occurs whether tubulin mRNA remains on polysomes or is released. These results are not consistent with the predictions based on our cotranslational model, although they do not invalidate the model (see below).

The protection of tubulin mRNA from decay by protein synthesis inhibitors in embryos treated with colcemid might result if the inhibitors were to interfere with the depolymerization of microtubules; this does not occur in CHO cells (33) and is unlikely to occur in sea urchin embryos for the following reasons. Treatment of embryos with either nocodazole or low concentrations (10 μM) of vinblastine also results in depolymerization of microtubules and a rapid decline in cytoplasmic tubulin RNA (21). Emetine treatment prevented this decline, and puromycin treatment prevented the decline of tubulin RNA in embryos treated with low concentrations of vinblastine (data not shown). Since colcemid, nocodazole, and vinblastine have different mechanisms of action, the rescue of tubulin mRNA by inhibitors of protein synthesis is unlikely to be due to their interfering with the action of the microtubule-depolymerizing agents that were used.

TABLE 1. Stability of tubulin RNA in the presence of inhibitors of protein synthesis[a]

Treatment[b]	Half-life of:	
	β-Tubulin RNA	α-Tubulin RNA (1.75 kilobases)
A	162 min	150 min
AC	33 min	20 min
AP, APC	120 min	111 min
AE	12 h	7.5 h

[a] Tubulin mRNAs were analyzed by RNA gel blot analysis, and half-lives were estimated from semilogarithmic plots of densitometric data, as shown in Fig. 5.

[b] Treatments: A, control embryos treated only with actinomycin D; AC, colcemid-treated embryos; AP, puromycin-treated embryos; APC, puromycin- and colcemid-treated embryos; AE, emetine-treated embryos.

The depolymerization of microtubules by colcemid treatment in the presence of inhibitors of protein synthesis was shown directly. Embryos were incubated with [^{35}S]methionine and extracted under conditions which stabilize microtubules. Tubulin was immunoprecipitated from the soluble extract and analyzed by electrophoresis and autoradiography. Colcemid treatment increased the amount of soluble tubulin to a similar extent in control embryos and those treated with emetine or pactamycin (Fig. 6C).

Inhibition of protein synthesis rescues tubulin mRNA in embryos treated with colcemid. The normal level of tubulin mRNA could be restored in embryos treated with microtubule-depolymerizing agents for several hours by inhibition of protein synthesis (Fig. 7). Early plutei were treated with colcemid for 2.5 h, which resulted in a loss of more than 70% of the tubulin mRNA. Initiation of puromycin treatment at this time resulted, after a lag of 3 h, in a rapid reaccumulation of β-tubulin RNA (as well as α-tubulin RNA; data not shown) to nearly normal levels after 6 h. Treatment with emetine also rescued tubulin mRNA in embryos that were treated with colcemid (data not shown). The response was faster for emetine than it was for puromycin, and by 6 h tubulin RNA accumulated to a higher level than that in control embryos that were not treated with colcemid or emetine. The more rapid and extensive effects on protein synthesis of emetine treatment compared with that of puromycin treatment may be due to its more rapid and com-

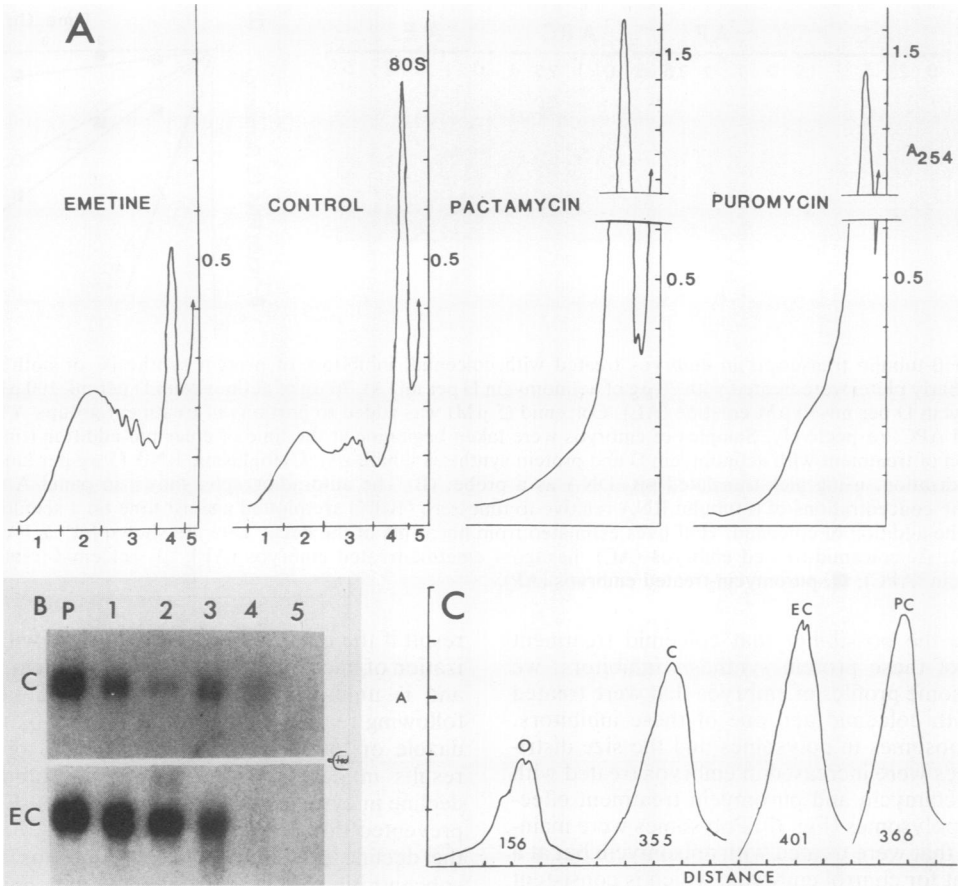

FIG. 6. Effects of colcemid and inhibitors of protein synthesis on polysomes and microtubules. (A) Prism-stage embryos were treated for 2.5 h with 5 μM colcemid alone (control) or in combination with the standard concentration of one of the protein synthesis inhibitors: emetine, pactamycin, or puromycin. The distribution of polysomes in cytoplasmic extracts was analyzed by sucrose gradient centrifugation. The A_{254} is shown for fractions taken for the analysis of tubulin RNA shown in panel B. (B) RNA was extracted from each fraction of the sucrose gradient and from the pellet and β-tubulin RNA analyzed by gel blot analysis. Lane numbers correspond to fraction numbers in panel A, while lane P corresponds to RNA extracted from the polysome pellet; equal fractions of RNA were loaded onto each lane. Colcemid treatment reduced the level of tubulin mRNA in these embryos, and emetine treatment reversed this effect. EC, Emetine and colcemid treatment; C, colcemid treatment. (C) Late gastrula-stage embryos were incubated with [^{35}S]methionine for 2 h and chased by return to seawater for 8 h. They were then treated with either colcemid or colcemid in combination with emetine or pactamycin at the standard concentrations for 2.5 h. Cytoplasmic extracts were prepared under conditions which stabilize microtubules; and tubulin was immunoprecipitated, separated by electrophoresis on a polyacrylamide gel containing sodium dodecyl sulfate, and analyzed by autoradiography. Densitometric traces were prepared by using a laser densitometer (LKB Instruments, Inc., Rockville, Md.) and are shown; the integrated areas under each curve (absorbance × distance) are shown by the numbers. O, No colcemid; C, colcemid; EC, colcemid and emetine; PC, colcemid and pactamycin.

plete inhibition of protein synthesis (Fig. 1) and its greater stabilization of tubulin mRNA (Fig. 5). These observations indicate that the continuation of protein synthesis is required to maintain the destabilization of tubulin mRNA by an increased level of unpolymerized tubulin. When embryos were removed from colcemid after 2.5 h of treatment, the level of tubulin mRNA remained low for at least 6 h. The effects of colcemid on microtubules appeared to be irreversible or only slowly reversible.

The restoration of tubulin RNA prevalence by inhibition of protein synthesis in embryos treated with colcemid is consistent with our conclusion that colcemid treatment does not result in inhibition of tubulin gene transcription (21). Additional evidence for this conclusion is that the prevalence of tubulin transcripts in nuclear RNA from colcemid-treated or untreated embryos was similar (data not shown).

Precocious accumulation of tubulin RNA is induced by inhibition of protein synthesis. In *L. pictus* embryos, tubulin RNA remains at a constant low prevalence during the period of early cleavage following fertilization, but it begins to accumulate at the time of ciliogenesis shortly before hatching; this is accompanied by a change in the size distribution of transcripts and an increase in tubulin gene transcription; the prevalence of cytoplasmic tubulin RNA then slightly declines until the mid-gastrula stage, when it begins to accumulate again (2, 21; Gong and Brandhorst, submitted). We have proposed that following hatching the synthesis of tubulin is autogenously regulated at the level of tubulin mRNA stability (21). Several observations are consistent with this model. The rate of synthesis of tubulin RNA per embryo changes little after hatching; the rate of decay of tubulin mRNA in the presence of actinomycin D decreases during later embryogenesis; the sensitivity of loss of tubulin RNA to colcemid treatment increases during development, while treatment of embryos with the microtubule-stabilizing agent taxol at the gastrula stage but not at pluteus stages results in the elevation of tubulin mRNA prevalence; and the

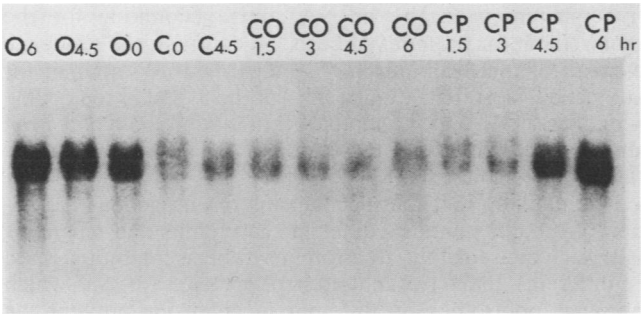

FIG. 7. Restoration of β-tubulin mRNA prevalence in colcemid-treated embryos by inhibition of protein synthesis. Early pluteus embryos were treated with 5 μM colcemid for 2.5 h and divided into three groups. The first group continued colcemid treatment (C); for the second group, colcemid was removed by extensive washes through 73-μm nylon mesh, and the embryos were incubated in normal seawater (CO); 100 μM puromycin was added to the third group (CP). Cytoplasmic RNA (5 μg loaded into each lane) was analyzed by RNA gel blot hybridization by using nick-translated $p\beta_2$ DNA. Numbers at the top of each lane correspond to the time (in hours) after time zero, the time of addition of puromycin, or the time of removal of colcemid; O, untreated embryos.

level of unpolymerized tubulin declines substantially following hatching (Gong and Brandhorst, submitted and in press).

If autogenous regulation of mRNA stability is responsible for the changing prevalence of the tubulin mRNA during embryogenesis, inhibition of protein synthesis should stabilize tubulin mRNA and the effect on tubulin mRNA prevalence should change ontogenetically. The effects of the four inhibitors of protein synthesis on β-tubulin RNA prevalence are shown in Fig. 8. In rapidly cleaving embryos, there was no effect of inhibition of protein synthesis on tubulin RNA prevalence; this was expected since the transcriptional activity of tubulin genes is very low in these embryos (26; Gong and Brandhorst, submitted). The inhibition of protein synthesis in early gastrulae resulted in extensive increases in the prevalence of tubulin mRNA. Inhibition of protein synthesis in plutei had little effect on the prevalence of tubulin RNA (Fig. 3), which was expected if the RNA was already almost maximally stable. Partial inhibition of protein synthesis by low levels of pactamycin did not result in precocious accumulation of tubulin mRNA in gastrulae (data not shown).

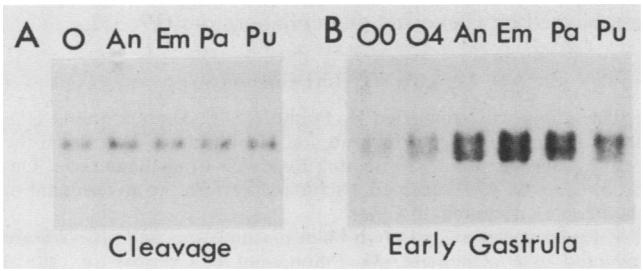

FIG. 8. Effects of protein synthesis inhibition on accumulation of β-tubulin RNA in the cleavage stage and early gastrula embryos. Embryos at the cleavage stage (A) or at the early gastrula stage (B) were treated with 30 μM anisomycin (An), 50 μM emetine (Em), 4 μg of pactamycin (Pa) per ml, or 100 μM puromycin (Pu) for 4 h, beginning at 5 or 20 h after fertilization, respectively. RNA gel blots of cytoplasmic RNA were hybridized to a β-tubulin-coding sequence RNA (5 μg per lane) probe transcribed from $b\beta_2T7c$. (A) O, Untreated control embryos. The prevalence of tubulin RNA is constant during the first 10 h of development (2, 21). (B) O0 and O4, Untreated control embryos at the time of the addition of an inhibitor of protein synthesis or 4 h later, respectively.

DISCUSSION

Each of the four inhibitors used in this investigation has a distinct mode of action (for a review, see reference 24). Puromycin is structurally analogous to the aminoacyl-adenyl terminus of aminoacyl-tRNA and can be incorporated into peptides, resulting in the premature termination and dissociation of polysomes. Pactamycin inhibits the formation of the initiation complex. Both emetine and anisomycin inhibit peptide chain elongation, resulting in the "freezing" of polysomes, but they act by different mechanisms. Anisomycin inhibits peptide bond formation through its effect on peptidyl transferase, while emetine primarily inhibits translocation. Thus, polysomes are maintained and stabilized in the presence of anisomycin or emetine and are dissociated in the presence of puromycin or pactamycin. These effects have been demonstrated directly in sea urchin embryos for emetine (27, 28) and pactamycin (17) at the concentrations used in this investigation. Moreover, in this investigation we confirmed the effects of these inhibitors on polysomes of embryos treated with colcemid and observed that tubulin mRNA behaves in the same way as the general population of mRNA in embryos treated with these agents.

The increase in stability of tubulin mRNA in embryos treated with emetine and the slight decline in embryos treated with puromycin (Fig. 5 and Table 1) suggest that the freezing of polysomes may increase the stability of tubulin mRNA, while release from polysomes may destabilize it; this has been proposed for mRNAs in other cells (29, 42). However, treatment with the standard concentration of pactamycin also increased the stability of tubulin mRNA in another experiment, while treatment with anisomycin did not (data not shown). The most effective inhibitors of protein synthesis, emetine and pactamycin, had the greatest stabilizing effect on tubulin mRNA. It was clear from the use of these agents at lower concentrations that their effects on the stability of tubulin mRNA require substantial inhibition of protein synthesis (Z. Gong, Ph.D. thesis, McGill University, Montreal Quebec, Canada, 1987). The differences in the effects of these inhibitors on the stability of tubulin mRNA correlated well with the differences in the extent of inhibition of protein synthesis and the kinetics of its decline.

The effects of inhibitors of protein synthesis on the autoregulation of tubulin synthesis in mammalian cells have been reported recently by Pachter et al. (33). While treatment of cultured CHO cells with puromycin or pactamycin prevented the destabilization of β-tubulin mRNA after treatment with colchicine, treatment with cycloheximide did not. Since cycloheximide is an inhibitor of peptide elongation via its effect on peptidyl transferase, this result conflicts with our observations on the use of emetine and anisomycin. The reason for this discrepancy remains unclear. Pachter et al. (33) did not report on the use of emetine or anisomycin, and we have been unable to compare experimental results directly because cycloheximide is a poor inhibitor of protein synthesis in sea urchins (27) and had no effect on tubulin mRNA levels. Cycloheximide is considerably more effective when it is applied to mammalian cells; the inhibition of amino acid incorporation in CHO cells was stated by Pachter et al. (33) to be 90% or more. While 90% inhibition by emetine or anisomycin resulted in substantial stabilization of tubulin mRNA in sea urchin embryos, it is possible that in CHO cells tubulin mRNA destabilization is less sensitive to inhibition of peptide elongation.

Pachter et al. (33) concluded that autogenous regulation of tubulin mRNA stability is independent of ongoing protein synthesis. In addition, they demonstrated that premature termination of peptide elongation resulting from the introduction of a nonsense codon near the 5′ end of the coding sequence of the β-tubulin mRNA prevents the decline in cytoplasmic transcript prevalence in cells treated with colchicine. This elegant experiment was interpreted to indicate that tubulin mRNA must reside in polysomes to be sensitive to autogenous destabilization by elevated levels of unpolymerized tubulin. An alternative interpretation, also consistent with the data that they presented, is that the premature termination of translation and release of the ribosome from the tubulin mRNA results in the destabilization of this tubulin mRNA, irrespective of the level of unpolymerized tubulin, masking the autoregulatory effect. No information was provided about the stability of this altered tubulin mRNA. Pachter et al. (33) presented cotranslational models consistent with their interpretations of their experimental results. They proposed that soluble tubulin binds either to the tubulin mRNA or, more likely, to the nascent tubulin protein, resulting in a direct destabilization of the tubulin mRNA or translational arrest, increasing the susceptibility of the mRNA to degradation.

While it is obvious that the differential stability of mRNA plays an important role in the regulation of mRNA prevalence and protein synthesis, there is little information about the mechanisms which control mRNA stability. Several reports indicate that the rapid decay of some mRNAs requires continuing protein synthesis. These include the rapid decay of some histone mRNAs at the end of the S phase or after inhibition of DNA synthesis (4, 7, 16, 22, 39, 41, 43), mRNAs encoding flagellar proteins including tubulins after deflagellation of *Chlamydomonas reinhardi* (3), c-*myc* mRNA in some normal and transformed human cells (15), interferon mRNA after induction (35), interleukin-2 mRNA (18), and mRNA of the muscle isoform of creatine kinase (40). All of these mRNAs accumulate significantly under some circumstances but are destabilized under other circumstances, a process that requires protein synthesis. Some share an AU-rich tract near their 3′ end (38). Both α- and β-tubulin mRNAs from the sea urchin *L. pictus* are enriched in AU nucleotide sequences at their 3′ ends (1). It has been frequently proposed that destabilization of these mRNAs requires the ongoing synthesis of a labile protein effector, possibly a nuclease.

The role of protein synthesis in the destabilization of cell cycle-regulated histone mRNAs has been investigated intensively. Inhibition of protein synthesis with agents which either stabilize or dissociate polysomes prevents this destabilization (4, 7, 16, 22, 39, 41, 43). These observations are consistent with the requirement for a labile protein effector in mRNA destabilization (4, 22, 39, 43). Alternatively, it has been proposed that inhibition of protein synthesis prevents the accumulation of excess histone protein after the cessation of DNA synthesis, preventing an autogenous destabilization of the histone mRNA (37). Recently, it has been shown, by using histone mRNAs with in-frame termination codons, that destabilization of the histone mRNA requires that it be translated to within 300 nucleotides of a critical hairpin loop at the 3′ end, which is necessary for the destabilization of the mRNA in the absence of DNA synthesis; prematurely terminated histone mRNAs are stabilized (23, 31). This suggests that the degradation of the histone mRNA is coupled to its translation (not simply its residence in polysomes), which might provide access of the degradative machinery to the mRNA, thus accounting for the requirement for protein synthesis. In contrast, the sequence element of the β-tubulin mRNA identified as sufficient for autogenous destabilization resides at the 5′ end of the coding sequence (19), and premature termination has not been shown to stabilize the mRNA (as pointed out above, it may result in destabilization).

It is unlikely that the mechanisms of autogenous regulation of tubulin synthesis differ in sea urchins and mammals (although the sensitivity to protein synthesis inhibition could be different). Since two inhibitors of protein synthesis which preserve polysomes by different mechanisms prevent the destabilization of tubulin mRNA in sea urchin embryos, it is most reasonable to conclude that active destabilization of tubulin mRNA, as well as a variety of other mRNAs, requires at least a low level of protein synthetic activity. Several interpretations of this conclusion are possible which are not contravened by the other experimental results reported by Pachter et al. (33). The autogenous destabilization of tubulin mRNA could require a labile protein effector. The effector could be a nuclease or could bind to the target mRNA or nascent polypeptide to form a complex with increased sensitivity to nuclease. In this case the cotranslational model for autogenous regulation of protein synthesis proposed by Brandhorst et al. (8, 9) could be valid, but it could be obscured by the loss of the labile effector after inhibition of protein synthesis.

An alternative interpretation is that autogenous destabilization of tubulin mRNA requires that it be actively translated by ribosomes transiting along it. The stabilization of tubulin mRNAs by inhibitors of protein synthesis might then be the result of prevention of a change in mRNA conformation mediated by the ribosome as a cofactor, or by inhibiting access of the degradative machinery, which possibly resides on the ribosome (33), to the mRNA, as proposed previously for histone (23) and tubulin mRNAs (33). The residual rate of peptide elongation in CHO cells treated with cycloheximide might be sufficient to allow this access. Finally, it is possible that inhibition of protein synthesis results in such extensive changes in cellular organization and physiology that post-transcriptional gene regulatory mechanisms are abrogated. These alternative interpretations of the requirement for ongoing protein synthesis are not mutually exclusive, and it should be possible to design experiments to distinguish among them, making use of the elegant experimental system established by Cleveland and collaborators (19, 33).

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