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Autogenous regulation of tubulin synthesis *via* RNA stability during sea urchin embryogenesis

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Summary

When pluteus embryos of *Lytechinus pictus* were treated with colcemid, the incorporation of [^{35}S]methionine into tubulin declined by 5- to 15-fold within 4 h. This was mostly accounted for by a rapid decline in the concentration of α- and β-tubulin mRNA in the cytoplasm. Treatment with other microtubule depolymerizing agents (colchicine, nocodazole, low concentrations of vinblastine) had similar effects. Treatment of embryos with the microtubule-stabilizing agent, taxol, or high concentrations of vinblastine resulted in increased synthesis of tubulin. The concentration of tubulin mRNA increases during development and becomes increasingly sensitive to colcemid and decreasingly sensitive to taxol. The transcriptional activity of tubulin genes, estimated by an RNA run-on assay in isolated nuclei, was not altered after colcemid treatment. On the other hand, the rate of decay of tubulin mRNA in prism embryos treated with actinomycin D was increased several fold by colcemid treatment, while taxol treatment led to an increased half-life of tubulin mRNA. These observations suggest that tubulin synthesis is autogenously regulated at the level of mRNA stability by the level of unpolymerized tubulin. The increasing polymerization of microtubules and declining level of unpolymerized tubulin during embryogenesis would result in a stabilization of tubulin mRNA accounting for the increasing concentration of tubulin mRNA and rate of tubulin synthesis, as well as the increasing sensitivity of tubulin synthesis to microtubule-depolymerizing agents. Evidence is also presented for a rapid influence of the level of unpolymerized tubulin on the efficiency of translation of tubulin mRNA.

Key words: tubulin, microtubules, mRNA stability, sea urchin embryo, translational regulation, colcemid, taxol.

Introduction

The sea urchin egg contains stores of mRNA utilized during early embryogenesis for protein synthesis as well as proteins assembled during embryogenesis into organelles. We are interested in the expression of tubulin genes in eggs and embryos as a prototype for the regulation of expression of structural genes during embryogenesis. Sea urchin eggs contain a small store of tubulin mRNAs (Raff *et al.* 1972; Alexandraki & Ruderman, 1985*a*,*b*), large amounts of tubulin (Raff *et al.* 1971; Stephens, 1977), but few polymerized microtubules (Harris *et al.* 1980; Bestor & Schatten, 1981; Schatten *et al.* 1982). During embryogenesis, maternal tubulin is assembled into microtubules of the cytoskeleton, mitotic apparati and cilia (Stephens, 1972, 1977; Raff, 1975; Bibring & Baxandall, 1977, 1981; Harris *et al.* 1980). The amount of tubulin mRNA and the rate of tubulin protein synthesis increase during embryogenesis (Alexandraki & Ruderman, 1985*a*,*b*; Bédard & Brandhorst, 1983). The mass of tubulin changes little during embryogenesis (Raff, 1975; Bédard & Brandhorst, 1983; Gong & Brandhorst, unpublished data) and there is no net change in mass of protein or RNA per embryo.

The expression of tubulin genes appears to be autogenously regulated in cultured mammalian cells *via* the level of unpolymerized dimers of α- and β-tubulin, the subunits of microtubules. Agents that cause depolymerization of microtubules, thereby elevating the concentration of tubulin subunits, cause a rapid decline in the rate of tubulin synthesis, due to a decrease in the cellular concentration of tubulin mRNAs (Ben-Ze'ev *et al.* 1979; Cleveland *et al.* 1981).

A similar effect results from elevation of unpolymerized tubulin by microinjection (Cleveland *et al.* 1983). The rapid loss of tubulin mRNA in the presence of colchicine is not accompanied by a detectable change in the rate of tubulin gene transcription (Cleveland & Havercroft, 1983) and occurs in anucleate cytoplasts (Caron *et al.* 1985*b*; Pittenger & Cleveland, 1985). Thus, the apparently autogenous regulation of tubulin synthesis operates, at least in large part, through the control of the stability of tubulin mRNA in mammalian cells.

The expression of genes encoding structural proteins comprising cellular organelles is likely to be as finely regulated during embryogenesis as in somatic cells. Compared to somatic cells, the sea urchin embryo presents special problems for the regulation of synthesis of tubulin and assembly into microtubules because of the initially high concentration of unpolymerized tubulin. Autogenous regulatory mechanisms may provide a particularly useful mechanism to the developing embryo for controlling gene expression during its rapid conversion from a storehouse of unassembled components to a complex form consisting of differentiated cells characterized by unique organelle configurations assembled from stored maternal as well as newly synthesized components (Brandhorst *et al.* 1986).

We report here that depolymerization of microtubules in advanced embryos leads to a rapid decline in concentration of tubulin mRNA and rate of tubulin synthesis. This response is not observed during early development but is gradually acquired during embryogenesis. The reduction in tubulin synthesis is largely the result of destabilization of tubulin mRNA, though the efficiency of translation of tubulin mRNA also appears to rapidly respond to changes in the level of unpolymerized tubulin. We present evidence that autogenous regulation at the level of tubulin mRNA stability is a major process whereby tubulin gene expression is controlled during embryogenesis.

Materials and methods

Materials

L. pictus sea urchins were purchased from Marinus, Long Beach, CA. Colcemid (demecolchicine), colchicine, vinblastine, nocodazole and actinomycin D were purchased from Sigma; dimethylsulphoxide (DMSO) and glycerol from Fisher Scientific (Canada). Taxol was kindly given by the US National Cancer Institute and was stored as a 10 mM-stock in DMSO at −80°C. Anti-tubulin antiserum (whole serum), raised in rabbits against sea urchin tubulins, was purchased from Polysciences Inc., protein A–Sepharose CL-4B from Pharmacia. [^{35}S]methionine (>800 Ci mmol^{-1}) was purchased from Amersham. [8-^{3}H]uridine (50 Ci mmol^{-1}) was purchased from ICN. The α-^{32}P-UTP (410 Ci mmol^{-1}) and α-^{32}P-dCTP (>3000 Ci mmol^{-1}) were purchased from Amersham or ICN. Nick-translation kits were purchased from BRL and used according to their instructions. The two tubulin cDNA plasmids used, pα_2 and pβ_2, were the kind gift of Dr Joan Ruderman; they correspond to the 3′ noncoding and partial coding sequences of α- and β-tubulins, respectively (Alexandraki & Ruderman, 1983). The *Pst*I insertion site was regenerated in both instances, allowing for isolation of the inserts.

Unless otherwise noted the following concentrations were used: colcemid, 5 μM; colchicine, 200 μM; nododazole, 10 μM; taxol, 10 μM. At these concentrations, all these agents rapidly inhibited cleavage and are effective antitubule agents in a variety of other cell types. This concentration of colcemid caused depolymerization of the mitotic spindle in sea urchin embryos (Aronson & Inoue, 1970).

Culturing and labelling of embryos

Gametes were obtained and cultures established according to Klein, Harding & Barndhorst (1987). Embryos were cultured in artificial sea water (reagent grade) at 18°C with stirring. Under these conditions, hatching usually occurred at about 12 h after insemination, gastrulation began at 20 h, prisms were formed by 44 h and early plutei by 50 h. For labelling of proteins, embryos were suspended at no more than 10000 ml^{-1} and incubated with 100 μCi ml^{-1} [^{35}S]methionine for 0·5 or 1 h. The embryos were collected and washed by centrifugation through cold sea water and either lysed immediately or stored frozen at −80°C. Incorporation into protein was determined by precipitation with 15 % trichloracetic acid onto a Whatman GF/C filter and scintillation counting.

Protein electrophoresis and immunoprecipitation

Discontinuous electrophoresis in SDS and protein A–Sepharose immunoprecipitations were carried out as described previously (Gong & Brandhorst, 1987*a*). The amount of immunoprecipitated radioactive protein having a mobility corresponding to the tubulins was linearly related to the input as long as the reaction mix contained less than 9 μg total sea urchin protein (corresponding to 150 embryos). Readdition of the antiserum to the supernatant remaining after collection of the initial immunoprecipitate resulted in the precipitation of less than 5 % more material comigrating with tubulins. These observations indicate that the immunoprecipitation conditions used are satisfactory for a comparison of the relative rates of tubulin synthesis.

Purification and handling of RNA

Total cytoplasmic RNA was prepared by precipitation with LiCl from urea followed by phenol extraction as described (Gong & Brandhorst, 1987). Total RNA was prepared by differential precipitation from guanidine isothiocyanate and guanidine hydrochloride according to March *et al.* (1985). RNA was separated after denaturation in formamide/formaldehyde 1·2 % agarose gels in MOPS buffer containing 3·2 % formaldehyde according to Lehrach *et al.* (1977). The gel was stained with ethidium bromide and photographed over ultraviolet light, before being blotted onto GeneScreen or GeneScreenPlus filters (New England Nuclear) in 25 mM-phosphate buffer (pH 6·8). The filter was

baked for 2–4 h at 80 °C under vacuum. Filter hybridization was carried out as described by Conlon *et al.* (1987). Lanes compared were loaded with equal amounts of RNA, corresponding to equal numbers of embryos.

Run-on assay of transcriptional activity

Nuclei were prepared by a modification of the method of Morris & Marzluff (1983), as described by Conlon *et al.* (1987). RNA chain extension was according to Conlon *et al.* (1987) in the presence of α-^{32}P-UTP. Under these conditions there is strand-specific transcription of some genes. Run-on RNA was prepared from nuclei essentially as described by Groudine *et al.* (1981). Hybridization with chain-extended RNA (usually $1–2\times10^6$ cts min^{-1} in 1–1·5 ml) was carried out in a sealed microcentrifuge tube for 3 days; under our conditions filter-bound DNA is in great excess and the reaction approaches completion (Conlon *et al.* 1987).

Densitometry

Quantification of autoradiograms was by densitometry using a Biorad 1650 scanning densitometer connected to a Hewlett-Packard 3390A integrator. Multiple exposures were made to insure linearity. Gels and blots were stained with ethidium bromide; adjustments were made in densitometric quantification for observed variations in loading of lanes and blotting. Adjustments were not made for possible local variations in hybridization efficiency.

Results

The effects of anti-microtubule agents on tubulin synthesis and mRNA prevalence

As an initial effort to establish whether tubulin synthesis is autogenously regulated, *L. pictus* early plutei were treated with the microtubule-depolymerizing agent colcemid. Proteins were pulse labelled with [^{35}S]methionine and analysed by one- or two-dimensional gel electrophoresis. As shown in Fig. 1A, colcemid treatment caused a decline in the relative rates of amino acid incorporation into proteins comigrating with the major sperm tail and ciliary proteins, the tubulins. Few other proteins showed any decline in relative rate of synthesis; among them were the pair of spots marked 77K, having an apparent relative molecular mass of 77 000 (77K). Bloom *et al.* (1985) have identified a 77K protein in sea urchin eggs which associates with microtubules of the mitotic spindle but not microtubules of the cilia or sperm flagellae. The pair of 77K polypeptides shown in Fig. 1A does not correspond to this protein, based on dissimilarities in isoelectric points and immunological cross-reactivity (Z. Gong, Ph.D. Thesis, McGill University, 1987).

To facilitate quantification of the declining synthesis of tubulin, an antiserum raised to sea urchin tubulins was used to immunoprecipitate pulse-labelled proteins; autoradiographs of these proteins are shown in Fig. 1B. Densitometric analysis of the tubulin bands provided the time course of decay of tubulin synthesis shown by closed circles in Fig. 1D.

Caron *et al.* (1985*a*) have reported that in a hepatocyte cell line, but not in 3T3 fibroblasts, the stability of tubulin proteins is reduced by treatment with microtubule-depolymerizing agents. Sea urchin embryos were incubated with [^{35}S]methionine for 2 h and chased for 44 h with unlabelled methionine. Colcemid treatment during the chase did not result in an increased rate of decay of the labelled tubulins (data not shown). Thus, the decline in incorporation of methionine into tubulin in the presence or colcemid is not due to the destabilization of tubulin.

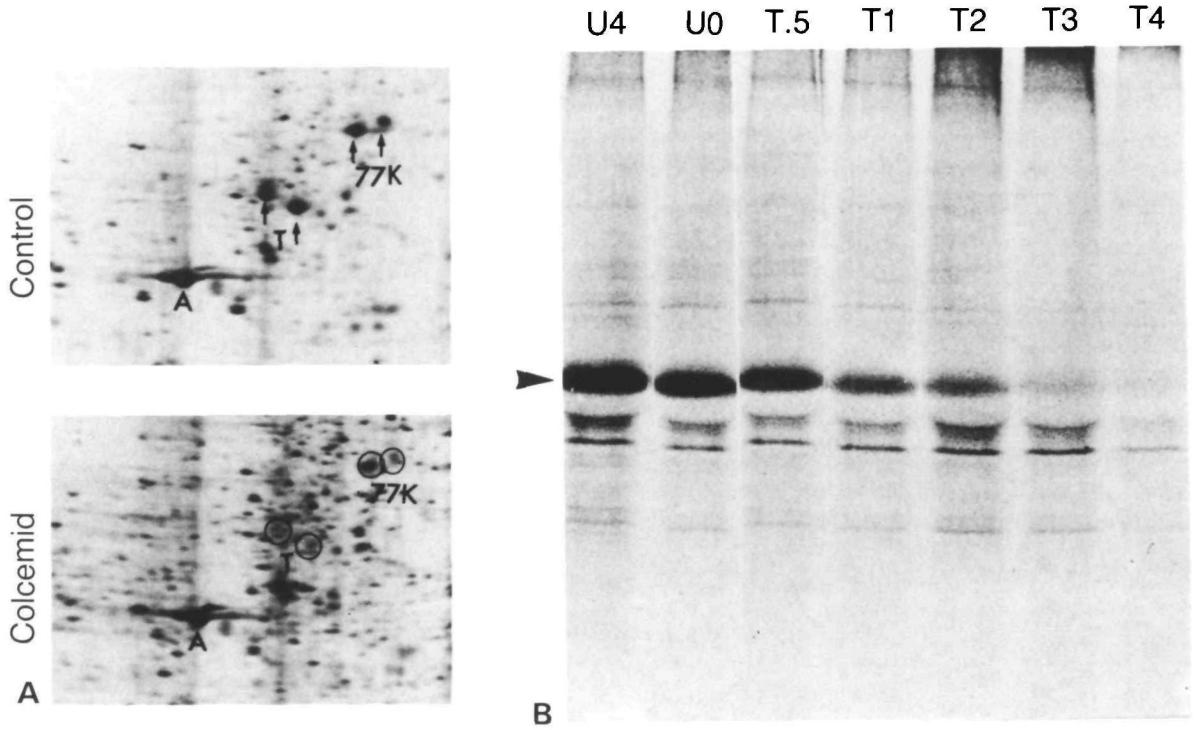
The behaviour of tubulin mRNA in these colcemid-treated embryos was analysed by RNA gel blot analyses as shown in Fig. 1C. Tubulin transcripts were detected by hybridization to two nick-translated tubulin cDNA clones, pα_2 and pβ_2. Clone pα_2 includes 438 bp of sequence encoding an α-tubulin, and 373 bp of 3′ noncoding sequence; pβ_2 contains 392 bp of sequence encoding a β-tubulin and 390 bp of 3′ noncoding sequence (Alexandraki & Ruderman, 1983). The clone pα_2 hybridized to several transcripts in eggs ranging from 1·75 to 3·6 kb, while the pβ_2 clone usually hybridized to two transcripts of 1·8 and 2·25 kb. Alexandraki & Ruderman (1985*a*) have also observed polymorphisms in the size distribution of tubulin transcripts of *L. pictus*. In agreement with Alexandraki & Ruderman (1985*b*), the level of tubulin mRNA began to increase before hatching and there was a change in transcript pattern: the size distribution of α-tubulin cytoplasmic transcripts became less complicated, with a 1·75 kb transcript becoming most prevalent. In late-stage embryos, three cytoplasmic β-tubulin transcripts ranging from 1·8 to 2·25 kb could usually be resolved.

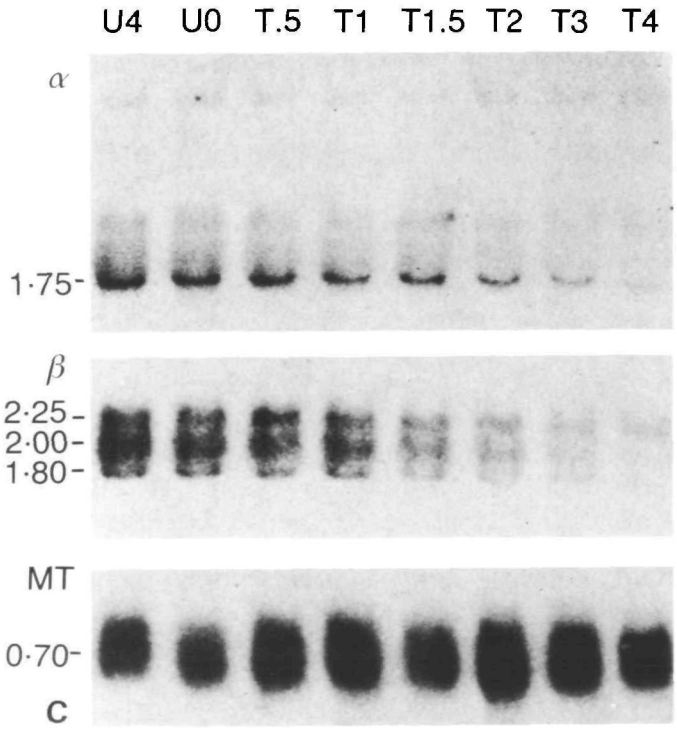
Cytoplasmic RNA was extracted from embryos treated with colcemid and equal amounts were separated by electrophoresis and blotted. Hybridization to α- and β-tubulin DNA probes is shown in Fig. 1C; tubulin RNA decayed rapidly and extensively during colcemid treatment. Hybridization of RNA on the same blot to a cDNA clone specific for metallothionein (MT) RNA showed that the mass of MT transcripts was not altered by colcemid treatment, and confirmed that the lanes compared in Fig. 1C had similar inputs of RNA. The time course of decay of tubulin transcripts was established by densitometry and is shown in Fig. 1D. For quantification, all three β-tubulin transcripts were included, but only the major (1·75 kb) α-tubulin transcript was scanned; the concentration of some of the minor α-tubulin transcripts appeared less sensitive to colcemid. Generally, colcemid treatment of early plutei usually resulted in a 10-fold reduction of cytoplasmic β-tubulin RNA

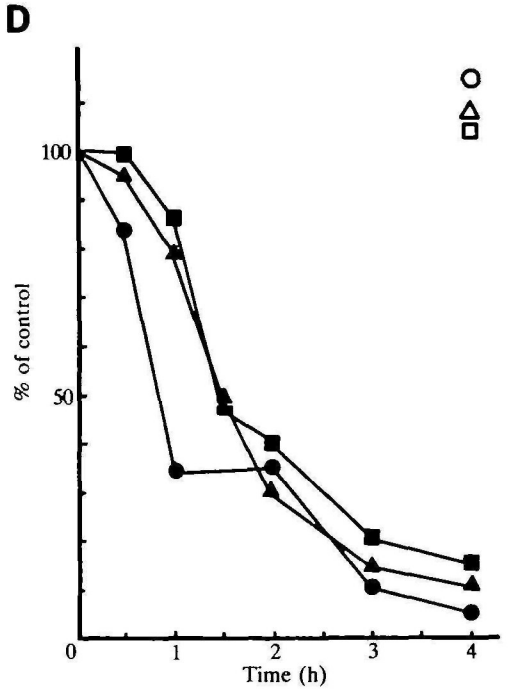
after 4–6 h, though this varied from 5- to 15-fold between experiments; somewhat less reduction was normally observed for α-tubulin RNA. A similar reduction in tubulin RNA was observed for total cellular RNA (data not shown).

The same batch of embryos was used for the assays of tubulin synthesis and tubulin mRNA shown in Fig. 1. It is clear that tubulin synthesis decreases in concert with the decline in mass of tubulin mRNA (though the rate of tubulin synthesis initially declined more rapidly). This excludes translational and post-translational levels of regulation as the principal mechanisms for the reduced rates of incorporation of methionine into tubulin after microtubule depolymerization.

The decline in level of tubulin mRNA in embryos treated with colcemid is consistent with an autoregulation of tubulin synthesis by unpolymerized tubulin







dimers, as proposed for various mammalian cells (Cleveland *et al.* 1981). The effects of other agents on tubulin synthesis and tubulin RNA levels were also consistent with this interpretation. Fig. 2 shows the relative rate of incorporation of methionine into tubulin in embryos treated with the microtubule-depolymerizing agents colcemid, colchicine, and nocodazole, as well as the microtubule-stabilizing agent taxol. Treatment of early plutei with nocodazole or colchicine resulted in decreases in tubulin synthesis similar to those observed for colcemid, while taxol treatment resulted in a small increase in tubulin synthesis. RNA gel blot analyses indicated that decreased concentrations of tubulin transcripts were responsible for the decreased synthesis of tubulin in embryos treated with colcemid, colchicine and nocodazole, while no corresponding increase in tubulin transcripts was observed in plutei treated with taxol.

Fig. 1. Changes in relative rate of tubulin synthesis and relative concentration of tubulin RNA following treatment of embryos with colcemid. Early plutei were treated with 5 μM-colcemid beginning at time 0 and aliquots were collected thereafter for analysis.
(A) Extracts containing similar quantities of radioactivity were separated by two-dimensional gel electrophoresis as described by Bédard & Brandhorst (1983). Sections of autoradiograms of these gels are shown for newly synthesized proteins of embryos treated for 4 h with colcemid and untreated controls. A, the actins; T, tubulins; 77K, a pair of spots showing a reduced relative rate of incorporation of methionine in embryos treated with colcemid. (B) Tubulin synthesis was monitored by incubating embryos with [^{35}S]methionine for 30 min beginning at the times shown. Protein extracts containing equal radioactivity were immunoprecipitated with an antiserum against sea urchin tubulins and the precipitates analysed by gel electrophoresis. The position of the tubulins on an autoradiograph of the gel is shown by the arrowhead. Numbers of lanes correspond to time (h) after initiation of colcemid treatment for treated (T) and untreated (U) embryos. (C) Cytoplasmic RNA was prepared, separated by electrophoresis through agarose gels, blotted onto GeneScreen and hybridized to nick-translated plasmid DNA corresponding to α- or β-tubulin. MT, clone 15D6 specific for metallothionein. Equal amounts (5 μg) of RNA were applied to each lane. Approximate transcript sizes (kb) are indicated on the left. Hours after initiation of colcemid treatment are shown for treated (T) and untreated (U) embryos.
(D) Densitometric quantification of data shown in panels A and B. Symbols indicate levels as a percentage relative to untreated embryos at time zero. Open symbols correspond to untreated embryos, which undergo a slight ontogenic increase in tubulin RNA and tubulin synthesis, while closed symbols correspond to embryos treated with colcemid. ●, tubulin synthesis; ■, β-tubulin RNA; △, α-tubulin RNA (1·75 kb transcript only).

Nocodazole and taxol were dissolved in DMSO before application to the embryos. Tubulin synthesis was barely altered by treatment of embryos with 0·1 % DMSO, the concentration resulting from application of these agents (data not shown). However, as shown in Fig. 2, treatment with 3 % DMSO led to increased tubulin synthesis. DMSO is frequently used to promote microtubule assembly *in vitro* (Himes *et al.* 1977; Robinson & Engleborough, 1982). Glycerol is another agent used to facilitate microtubule assembly *in vitro* and was also found to enhance tubulin synthesis in treated embryos. The elevated synthesis of tubulin in the presence of DMSO or glycerol might be the result of stabilization of microtubules in the treated embryos, but other mechanisms may be operating since both compounds can have a variety of effects on cellular activities.

Vinblastine is a microtubule-depolymerizing agent which causes tubulins to aggregate into paracrystals, presumably resulting in a decreased concentration of tubulin dimers (Bensch & Malawista, 1969; Bryan,

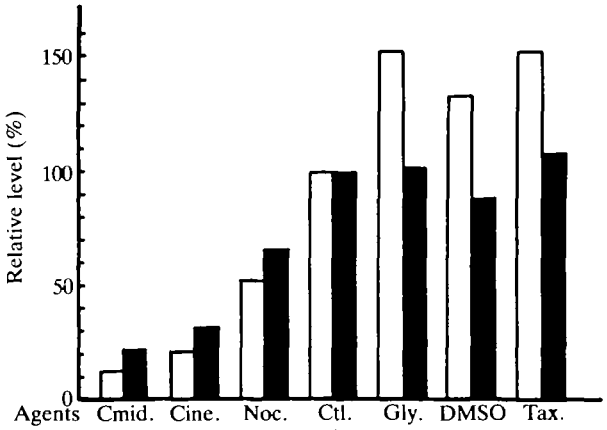


Fig. 2. Effects of treatment of embryos with various agents on tubulin synthesis and tubulin transcript prevalence. Early plutei were treated with the following agents: Cmid., 5 μM-colcemid; Cine., 200 μM-colchicine; Noc., 10 μM-nocodazole, ctl., untreated control embryos; gly., 1 % glycerol; DMSO, 3 % dimethylsulphoxide; Tax., 10 μM-taxol. Cytoplasmic RNA was prepared 4 h after initiation of treatment, or embryos were incubated with [^{35}S]methionine from 3·5 to 4·5 h after addition of the agents. Relative tubulin synthesis (open bars) was determined by densitometric analysis of autoradiographs of immunoprecipitated tubulins resolved by electrophoresis. Concentrations of β-tubulin RNA relative to untreated controls (solid bars) were estimated by densitometry of RNA gel blots. Incorporation into tubulin and β-tubulin transcript prevalence in untreated control embryos were set equal to 100 %. CTE, the coefficient of translational efficiency, is defined as the ratio of tubulin synthesis to the concentration of β-tubulin RNA in arbitrary units and is set equal to 1·00 in untreated control embryos; values are shown on the bottom line.

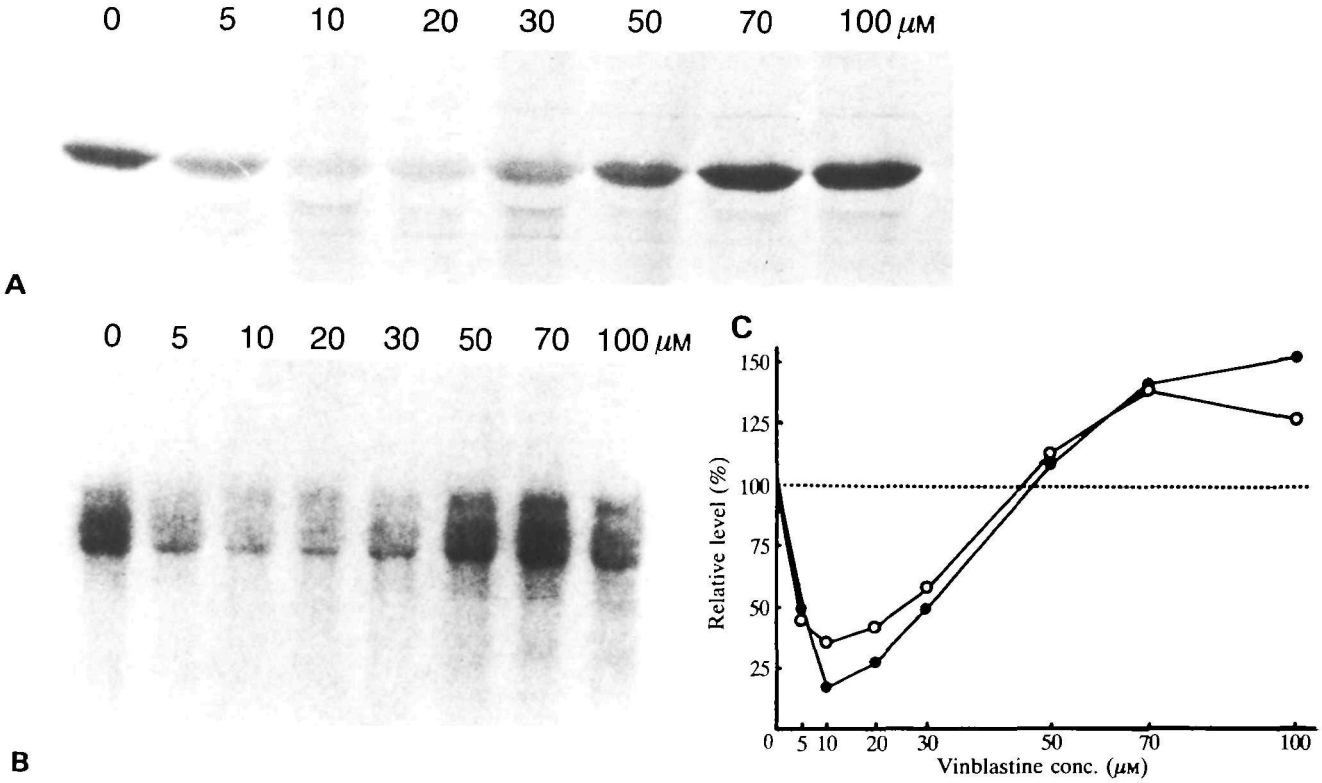


Fig. 3. Concentration-dependent effects of vinblastine on tubulin synthesis and RNA concentration. Early plutei were treated with various concentrations of vinblastine. Embryos were labelled with [^{35}S]methionine for 1 h beginning at 3·5 h; RNA was prepared from embryos 4 h after initiation of treatment. Corresponding concentrations of vinblastine are shown above each lane. (A) Analysis of immunoprecipitated tubulin was carried out as in Fig. 1. (B) RNA gel blot hybridization analysis of β-tubulin RNA was carried out as described for Fig. 1. (C) Densitometric quantification of data shown in panels A and B. ●, Rate of tubulin synthesis relative to untreated embryos; ○, concentration of β-tubulin RNA relative to untreated embryos.

1971). As shown in Fig. 3, the effects of vinblastine on tubulin synthesis in treated early plutei is concentration dependent. Treatment with vinblastine below 50 μM resulted in decreased tubulin synthesis (and a corresponding decline in tubulin transcript prevalence), while at higher concentrations a modest increase in tubulin synthesis was observed (with less increase in tubulin transcript prevalence). It has been reported that tubulins have two binding sites having different affinities for vinblastine (Bhattacharyya & Wolff, 1976). The higher affinity site has been proposed to be responsible for prevention of assembly of tubulins into microtubules, while the lower affinity site would be responsible for the aggregation of tubulins into paracrystals. Thus the application of vinblastine at low concentration should increase the concentration of unpolymerized tubulin, while higher concentrations of vinblastine should result in decreased concentrations of unpolymerized tubulin. Our observations on tubulin synthesis in embryos treated with these drugs are thus consistent with the model of autogenous regulation of tubulin synthesis by unpolymerized tubulin.

The effects on embryogenesis of these anti-microtubule drugs were monitored. In all instances, cleavage was inhibited, hatching was inhibited if applied shortly before hatching and gastrulation was inhibited if applied shortly before gastrulation. Treatment of embryos following gastrulation for 6 h did not result in abnormalities other than developmental retardation. Early embryos showed an increasing fraction of abnormal embryos depending on concentration of DMSO, while 1 % glycerol had little effect on early development except for slight retardation.

The sensitivity to anti-microtubule agents changes during embryonic development

The effect of colcemid treatment on the prevalence of tubulin mRNA at other stages of embryogenesis was assessed by RNA gel blot analyses. Fig. 4 shows the relative mass of cytoplasmic β-tubulin mRNA in control and colcemid-treated embryos. As reported by Alexandraki & Ruderman (1985*a*), the concentration of tubulin mRNA increased beginning before hatching, declined during gastrulation and increased again during late embryogenesis. Only more advanced embryos suffered a decline in tubulin mRNA

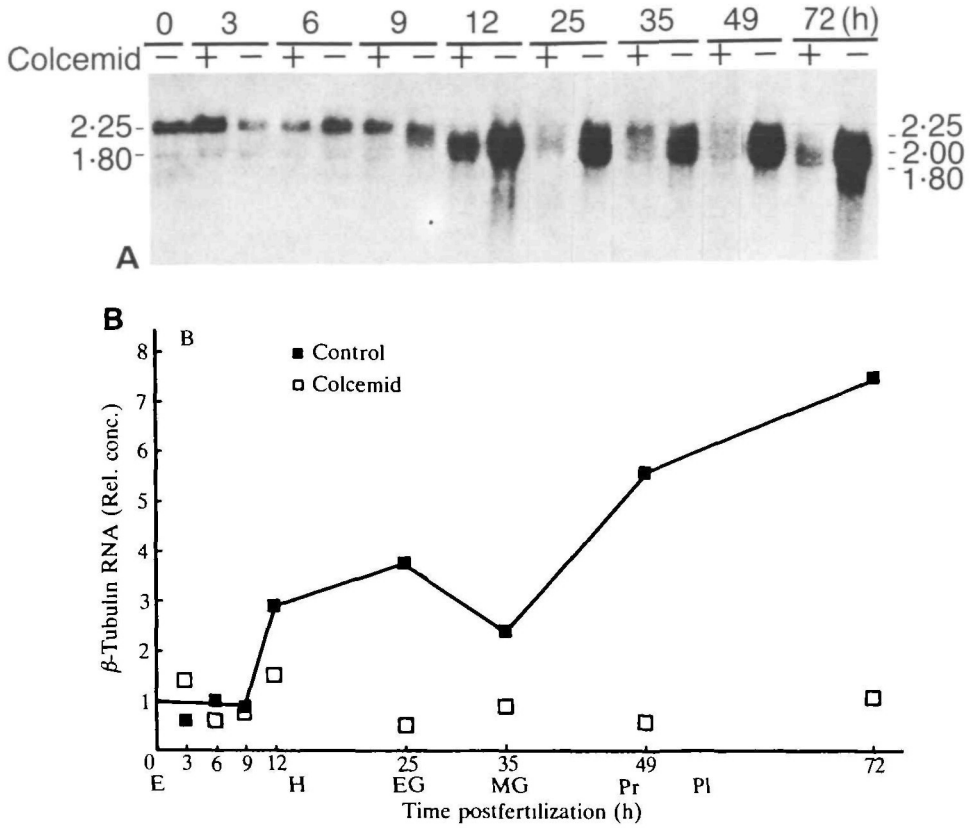


Fig. 4. Changes in tubulin transcript prevalence and sensitivity to colcemid during embryogenesis. Aliquots of a culture of embryos were removed at various times and treated for 3 h with 5 μM-colcemid. Cytoplasmic RNA was prepared from treated and untreated embryos and analysed by gel-blot hybridization to nick-translated plasmid DNA corresponding to β-tubulin. (A) Autoradiographs of β-tubulin RNA is treated (+) and untreated (−) embryos are shown. Times correspond to the time after fertilization when colcemid treatment was initiated. Numbers on the right and left correspond to estimated transcript sizes (kb). Each lane was loaded with the same amount (5 μg) of cytoplasmic RNA. (B) Densitometric quantification of data shown in panel A. The estimated concentration of β-tubulin transcripts is shown relative to the amount in eggs. ■, untreated embryos; □, embryos treated with colcemid. Ethidium bromide staining indicated that RNA in the lane corresponding to 12 h untreated embryos was overloaded; the densitometric value shown is from another blot of the same preparation of RNA. Developmental stages are indicated as follows: E, egg; H, hatching blastula; EG, early gastrula beginning invagination; MG, mid-gastrula; Pr, prism; Pl, pluteus.

levels in response to colcemid treatment; the fraction of tubulin mRNA lost increased as the mass of tubulin mRNA increased, while the level of tubulin mRNA after colcemid treatment was similar at all stages. Thus there is an ontogenetic acquisition of sensitivity of tubulin mRNA concentration to colcemid treatment; tubulin synthesis showed a similar pattern of ontogenetic sensitivity to colcemid (data not shown).

The ontogenetic sensitivity to the microtubule-stabilizing agent, taxol, showed the opposite pattern. As shown in Fig. 5, treatment of plutei with taxol resulted in increased synthesis of tubulin without a corresponding change in concentration of tubulin transcripts, suggesting a translationally mediated regulation of tubulin synthesis. On the other hand, when early gastrulae were treated with taxol (as well as glycerol or DMSO, data not shown), tubulin synthesis increased and the concentration of tubulin transcripts also increased, after a delay.

Evidence for post-transcriptional regulation of tubulin synthesis

An RNA chain extension assay in isolated nuclei was used to compare the transcriptional activities of tubulin genes in colcemid-treated and untreated embryos. Because nuclei isolated from plutei are considerably less active than nuclei prepared from earlier stages in incorporation of ^{3}H-UTP into RNA in this run-on assay (our observations; Uzman & Wilt, 1984), we used nuclei from gastrulae. Embryos were treated for 4 h with 5 μM-colcemid and nuclei were prepared from treated and untreated embryos; total incorporation into RNA was similar for treated and

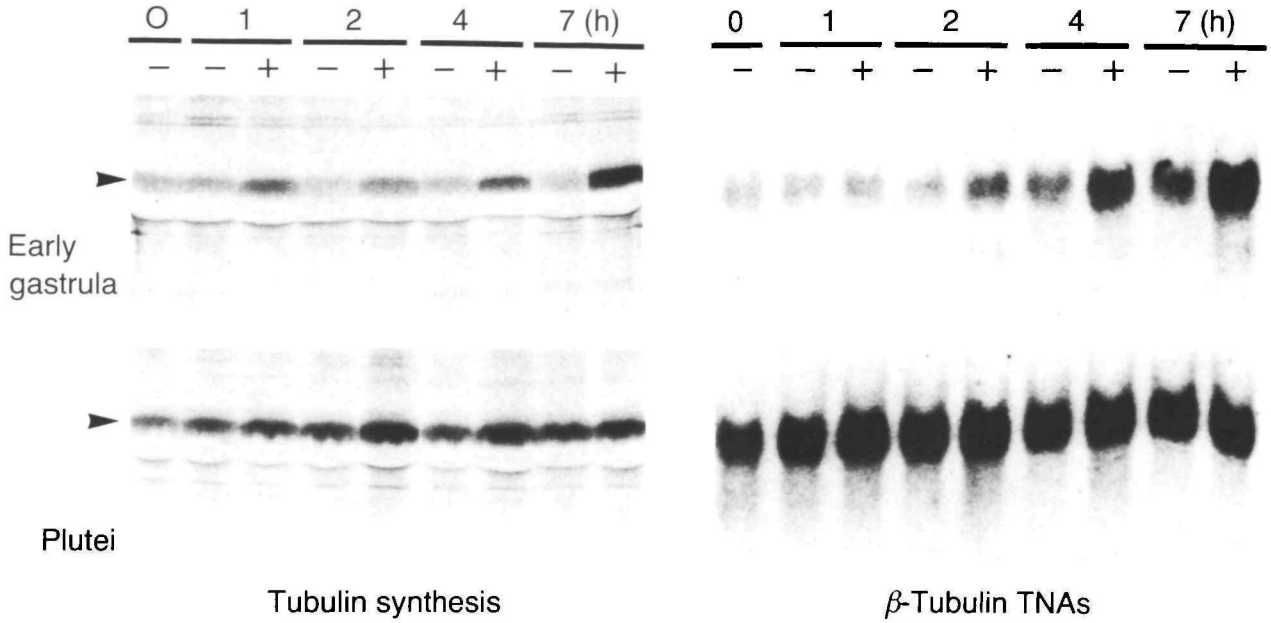


Fig. 5. Effects of taxol treatment on tubulin synthesis and tubulin transcript prevalence in gastrula and pluteus embryos. Early gastrulae (20 h postfertilization, A,B) or early plutei (44 h, C,D) of the same batch of embryos were incubated with 10 μM-taxol. The relative synthesis of tubulin (A,C) and prevalence of β-tubulin RNA (B,D) were monitored as described for Fig. 1. Times after the beginning of taxol treatment are shown. −, untreated embryos; +, embryos treated with taxol.

untreated nuclei. RNA labelled by chain extension was hybridized to excess $p\alpha_2$ or $p\beta_2$ plasmid DNA on filters.

Fig. 6A shows the results of a typical investigation. The fraction of RNA hybridizing to tubulin DNA was similar for nuclei prepared from treated or untreated embryos, indicating that the rates of tubulin gene transcription are similar. Also shown is the hybridization of run-on RNA to DNA of five nontubulin genes (including metallothionein) whose transcript prevalences are not sensitive to colcemid treatment. The minor variations in hybridization to these transcripts were used to normalize the efficiencies of hybridization to tubulin DNA for densitometric analysis. The relative rates of synthesis of α- and β-tubulin RNA in colcemid-treated embryos were 80 and 90 %, respectively, of that of control embryos. These alterations were not reproducible and are likely to be due to nonsystematic experimental errors. In the same batch of colcemid-treated embryos, the concentrations of α- and β-tubulin mRNAs in colcemid-treated embryos were 21 % and 9 %, respectively, of those in untreated controls (see Fig. 6B). These observations indicate that the decrease in concentration of cytoplasmic tubulin mRNAs in treated embryos is not due to a corresponding decrease in tubulin gene transcription.

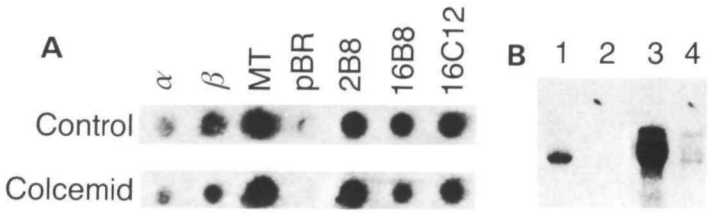


Fig. 6. Run-on assay of transcriptional activity of tubulin genes in embryos treated with colcemid. Gastrulae (24 h) were incubated with 5 μM-colcemid for 3 h. (A) Nuclei were isolated and incubated with ^{32}P-UTP under conditions allowing RNA chain extension. RNA was extracted from nuclei and 10^6 cts min^{-1} hybridized to a set of plasmid DNAs (5 μg) blotted onto GeneScreen. Autoradiographs are shown for RNA from embryos treated with colcemid or not treated (control). The identity of plasmid DNA dots is shown above the array: α, $p\alpha_2$ (α-tubulin); β, $p\beta_2$ (β-tubulin); MT, clone 15D6 specific for metallothionein; PBR, pBR322; 2B8, 16B8, and 16C12 are unidentified cDNA clones from an *L. pictus* gastrula cDNA library (Conlon *et al.* 1987). (B) Cytoplasmic RNA was prepared from embryos collected at the same time as nuclei were prepared for the investigation in panel A. The RNA was analysed for α- and β-tubulin transcripts by RNA gel blot hybridization. Lanes 1 and 2, probed with $p\alpha_2$ DNA; lanes 3 and 4, probed with $p\beta_2$ DNA (the same blot was used, but there were differences in specific activity of the probe). Lanes 1 and 3, untreated embryos; lanes 2 and 4, embryos treated with colcemid.

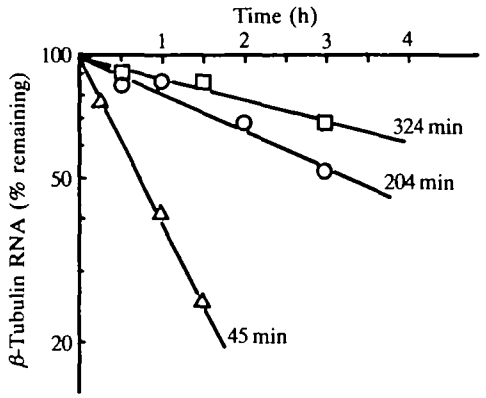


Fig. 7. Kinetics of decay of tubulin transcripts in embryos treated with colcemid or taxol. Prism-stage embryos were treated with 70 $\mu g\,ml^{-1}$ actinomycin D. Taxol (10 μM) was added to a portion of the embryos 0·5 h later. Colcemid (5 μM) was added to another portion 1 h after initiation of actinomycin D treatment. RNA was extracted and analysed by gel blot hybridization to pβ_2 plasmid DNA; autoradiographs were quantified by densitometry and the concentration of β-tubulin RNA relative to time zero plotted on a semilog scale for various times after initiation of treatment with taxol or colcemid. Incorporation of tritiated uridine into RNA was nearly maximally (70 %) inhibited by actinomycin D after 1 h of treatment. Time zero corresponds to 1 h after the initiation of treatment with actinomycin D for untreated and taxol-treated embryos but corresponds to 1·5 h for colcemid-treated embryos. Half-lives estimated from linear fits of the decay data are shown for each curve. ○, untreated embryos in actinomycin D; △, embryos treated with colcemid; □, embryos treated with taxol.

Evidence for destabilization of tubulin mRNA by elevated levels of unpolymerized tubulin

A rapid loss of tubulin mRNA is observed in anucleate cytoplasts of mammalian cells when treated with microtubule-depolymerizing agents (Caron *et al.* 1985*b*; Pittenger & Cleveland, 1985), indicating that tubulin mRNA is destabilized. Fig. 7 shows the decay of cytoplasmic tubulin RNA in prism-stage embryos treated with the inhibitor of RNA synthesis actinomycin D. RNA synthesis (as measured by incorporation of a 30 min pulse of tritiated uridine into RNA) was inhibited by at least 70 % 1 h after application of 70 $\mu g\,ml^{-1}$ actinomycin D and by 95 % after 3 h. Colcemid or taxol were added to cultures of embryos 1 or 1·5 h after the initiation of actinomycin D treatment, respectively. Cytoplasmic β-tubulin RNA decayed in untreated gastrulae with apparent first-order kinetics, indicating a half-life of 204 min, but were destabilized by colcemid treatment, showing an estimated half-life of 45 min. On the other hand, in the presence of taxol the stability of β-tubulin RNA was increased to 324 min. As shown in Table 1, in several repetitions of this experiment the stability of α- and β-tubulin mRNA varied somewhat, but was always decreased by several fold in embryos treated with colcemid.

Inhibition of RNA synthesis with actinomycin D might result in an alteration of the stability of RNA in general or tubulin mRNA in particular. Moreover, the failure to completely inhibit uridine incorporation suggests that some RNA synthesis continues, which would lead to an overestimate of RNA stability. However, the kinetics of decay of tubulin RNA during colcemid treatment when tubulin gene transcription is unaltered (e.g. as shown in Fig. 1) place an upper limit on the half-life of tubulin mRNA of 45–60 min. Moreover, the decline in tubulin mRNA stability is in reasonable agreement with the fraction of tubulin RNA lost after several hours of colcemid treatment, while the increased stability of tubulin RNA after taxol treatment is consistent with a comparable increase in concentration of tubulin RNA. It is thus likely that the kinetics of decay of tubulin RNA in embryos treated with actinomycin D are indicative of the relative stabilities of tubulin RNA under different experimental conditions.

Discussion

The results of our pharmacological experiments on sea urchin embryos of advanced stages are consistent with the model developed for mammalian somatic cells of autogenous regulation of tubulin synthesis by the concentration of unpolymerized tubulin. Treatments with agents that lead to an elevated level of unpolymerized tubulin led to a rapid decline in

Table 1. *Stability of tubulin RNA in embryos treated with colcemid*

	Time (h)	$t_{1/2}$ β-tubulin RNA (min)		$t_{1/2}$ α-tubulin RNA (1·75 kb) (min)	
		Control	Colcemid	Control	Colcemid
Exp. 1	44	204	45	124	42
Exp. 2	42	80	25	105	26
Exp. 3	45·5	162	33	150	20

Tubulin mRNAs were analysed by RNA gel blot analysis and half-lives ($t_{1/2}$) were estimated based on densitometric data.
* The time after fertilization when actinomycin D treatment began.

tubulin synthesis and concentration of cytoplasmic tubulin transcripts. Treatment with agents that lead to decreased concentration of depolymerized tubulin resulted in increased rates of tubulin synthesis, accompanied by increased concentrations of tubulin transcripts at gastrula (but not prism–pluteus) stage.

The rapid loss of tubulin mRNA after depolymerization of microtubules is not accompanied by a detectable change in the fraction of active polymerase II associated with tubulin genes, but, as shown in Fig. 7, is at least largely the result of a destabilization of cytoplasmic tubulin transcripts in embryos in which RNA synthesis is inhibited by actinomycin D. No alteration in transcript size or prevalence was observed by RNA gel blot analysis of nuclear RNA prepared from embryos treated with colcemid, suggesting that regulation is not at the level of nuclear processing (data not shown). Thus the regulation of tubulin synthesis by unpolymerized tubulin is largely at the level of tubulin mRNA stability.

Increased synthesis of tubulin was observed in prism–pluteus embryos the presence of several agents (taxol, glycerol, DMSO) without a corresponding increase in concentration of tubulin mRNA. This suggests that some regulation of tubulin synthesis may occur at the level of translation. Consistent with this is the observation that the decline in relative rate of tubulin synthesis in embryos treated with microtubule-depolymerizing agents is normally somewhat greater than the corresponding decrease in cytoplasmic tubulin transcript prevalence. This can be observed in the histogram of Fig. 4 in which a 'coefficient of translational efficiency' has been calculated from densitometric data. This coefficient is the ratio of tubulin protein synthesis to tubulin mRNA concentration in arbitrary units and is set equal to 1·0 in untreated control embryos. These coefficients were less than one in conditions in which tubulin synthesis declined and greater than one in conditions in which tubulin synthesis increased. Similar, or greater, changes in this coefficient were observed in similar experiments. The rate of tubulin synthesis responded more rapidly than tubulin RNA concentration to changes in the state of tubulin polymerization (see Figs 1C, 5) indicating a possible casual relationship between translational efficiency and mRNA stability.

Most translatable mRNA, including tubulin mRNA, is in polysomes at pluteus stage (Bédard & Brandhorst, 1986; Alexandraki & Ruderman, 1985*b*). Thus, an increase in translational activity is likely to be due to an increase in translational efficiency requiring increased peptide initiation (and, possibly, elongation); a decline in translational efficiency might result from a decreased rate of initiation or a change in the fraction of actively translated tubulin mRNA. Alexandraki & Ruderman (1985*b*) have reported that tubulin mRNA lags behind other mRNA in being recruited into polysomes following fertilization suggesting that a selective translational regulatory mechanism operates during early embryogenesis. The apparent regulation of tubulin synthesis at the level of translation is greatly overshadowed by the regulation of tubulin mRNA stability, except at advanced stages under conditions in which there is an increase in tubulin synthesis.

The sensitivity of tubulin synthesis to depolymerization of microtubules gradually increases during development: cleavage-stage embryos are insensitive to colcemid treatment while plutei are extremely sensitive. As shown in Fig. 4, after treatment of embryos with concentrations of colcemid sufficient to inhibit mitotic spindle formation the steady-state concentration of tubulin transcripts became nearly identical at all stages. This would be expected if the synthesis of tubulin during embryogenesis is normally autogenously regulated by the level of unpolymerized tubulin acting on the stability of tubulin mRNA. The mass of tubulin changes little during sea urchin embryogenesis (Raff, 1975; Bédard & Brandhorst, 1983; Gong & Brandhorst, unpublished data), but an extensive increase in microtubules occurs. Thus, the concentration of unpolymerized tubulin must decrease during embryogenesis, as confirmed by direct measurements (Gong & Brandhorst, unpublished data). According to the model of autogenous regulation, the stability of tubulin mRNA should increase during development, accounting, at least in part, for the ontogenetic increase in concentration of tubulin mRNA. As the level of unpolymerized tubulin mRNA decreases and the stability of tubulin mRNA increases, the sensitivity to depolymerization of microtubules should also increase. The concentration of tubulin mRNA in colcemid-treated embryos should thus reflect the minimum stability of tubulin mRNA (assuming that the rate of production of tubulin mRNA is similar at various stages; see below). The synthesis of tubulin in early embryos should be insensitive to microtubule depolymerizing agents because tubulin mRNA would be turning over with its minimum half-life, while the concentration of tubulin mRNA in plutei should be unaffected by microtubule-stabilizing agents because the stability of tubulin mRNA would already be at its maximum. These predictions are all consistent with our observations. This developmental regulatory profile is formally the same as that observed when cultured cells are subjected to partial depolymerization of microtubules, which results in intermediate levels of tubulin mRNA and tubulin synthesis (Caron *et al.* 1985*a*).

Consistent with this argument that autogenous regulation of tubulin synthesis operates throughout

embryogenesis is the observation that estimates of relative rates of tubulin gene transcription by run-on in isolated nuclei indicate that the rate of tubulin RNA synthesis per embryo peaks at about the time of hatching and slightly declines thereafter until pluteus stage (Gong & Brandhorst, unpublished data). Moreover, estimates of tubulin mRNA stability during development, based on the decay of tubulin transcripts after inhibition of RNA synthesis, indicate that the stability of tubulin mRNA increases several fold between gastrula and pluteus stages (Gong & Brandhorst, unpublished data). The increase in tubulin mRNA concentration after taxol treatment of gastrulae but not of plutei suggests that the stability of tubulin mRNA is maximal at pluteus stage and cannot increase in response to a decreased concentration of unpolymerized tubulin. The concentration of tubulin mRNA is rescued in prism embryos treated with colcemid by the inhibitors of protein synthesis puromycin, pactamycin, emetine and anisomycin; this is due to a stabilization of tubulin mRNA (Gong & Brandhorst, unpublished data). Treatment of earlier embryos with these inhibitors of protein synthesis leads to an increased accumulation of tubulin transcripts *via* the stabilization of tubulin mRNA.

Collectively these observations indicate that after hatching tubulin synthesis is largely controlled by the stability of tubulin mRNA, which in turn is regulated by the concentration of unpolymerized tubulin through a process requiring protein synthesis. The mechanism by which unpolymerized tubulin appears to destabilize tubulin mRNA remains a tantalizing problem.

According to the autoregulatory model, tubulin mRNA should be very unstable in early embryos and should thus decline rapidly after fertilization, since the rate of RNA synthesis per embryo is quite low (Wilt, 1970; Brandhorst, 1980); instead the mass remains constant. Our preliminary investigations indicate that tubulin mRNA in cleavage-stage embryos is quite stable, accounting for its constant concentration. It is possible that maternal tubulin mRNA is structurally distinct from the tubulin mRNA sensitive to levels of unpolymerized tubulin or that mRNA in general is relatively stable during cleavage stages. Alternatively, the autogenous regulatory system may not operate in early embryos, possibly because factors, such as a nuclease activity, required for rapid degradation of mRNA are lacking.

While posttranscriptional mechanisms are clearly very important for the ontogenetic regulation of tubulin gene expression in echinoid embryos, transcriptional regulation also has an important role. Before hatching, an increase in rate of tubulin gene transcription results in an increase in tubulin RNA concentration (Harlow & Nemer, 1987; Gong & Brandhorst, unpublished data). Moreover, deciliation of hatched embryos results in transient enhancement of the synthesis and accumulation of tubulin RNA (Gong & Brandhorst, 1987).

We have presented evidence for the importance of autogenous regulation of tubulin synthesis *via* RNA stability during echinoid embryogenesis. According to this model much of the tubulin required for embryonic development is stored in an unpolymerized form in the egg, but each cell of the advanced embryo can rapidly and specifically adjust to a demand for production of more microtubules by stabilizing tubulin mRNA, resulting in a rapid increase in tubulin mRNA concentration; thus stage- or tissue-specific regulation of the rate of tubulin gene transcription might be unnecessary. Autogenous regulation *via* RNA stability might be a particularly convenient and important mechanism for regulation of expression of genes encoding other structural proteins stored in the egg and assembled into complex structures during embryogenesis.

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