Coordinate and selective β -tubulin gene expression associated with cilium formation in sea urchin embryos

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β -Tubulin mRNAs associated with cilium formation in *Strongylocentrotus purpuratus* sea urchin embryos are expressed selectively from a multiple gene family. The accumulations of three β -tubulin mRNAs (β 1, β 2, and β 3) are temporally coordinated with ciliogenesis during blastula development and with the regeneration of cilia after their amputation. In contrast, another β -tubulin mRNA, β 4, is not induced in either case. The zinc-animalized embryo with its exaggerated blastula phenotype forms longer cilia through a protracted period of ciliogenesis, in which the β -tubulin mRNAs, principally β 1, accumulate to higher than normal levels. The rate of β -tubulin transcription per nucleus in the animalized embryo is greater than that of the normal embryo and is not changed through deciliation, although the tubulin mRNAs accumulate to higher levels. However, deciliation raises the β -tubulin transcription rate in the normal embryo to that in the animalized embryo. Thus, the induction of β -tubulin mRNA by cilium amputation is regulated transcriptionally in the normal embryo, but post-transcriptionally in the zinc-animalized embryos. Moreover, the β -tubulin genes that are expressed in association with cilium formation appear to be induced selectively within the framework of ectodermal cell-type specificity.

[Key Words: β -tubulin mRNAs; sea urchin embryo; cilia; zinc animalization; induced tubulin gene expression]

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Differential regulation of β -tubulin genes in the sea urchin embryo has been indicated by the quantitative distinctions among individual β -tubulin mRNAs in their developmental profiles and distributions among the tissues of the pluteus larva (Harlow and Nemer 1987). In the present study we focus on gene expression specifically associated with the construction of ciliary microtubules to determine whether there is either a selective or coordinate expression of individual genes in the ~12-gene β -tubulin family of *Strongylocentrotus purpuratus* (Harlow and Nemer 1987).

Control of the synthesis of axonemal proteins and the lengths of cilia and flagella has been studied through the simplifying use of unicellular organisms, as well as in the context of multicellular differentiation (for review, see Lefebvre and Rosenbaum 1986). In the sea urchin embryo, cilium formation occurs normally during development through the blastula stages and can be induced in response to cilium amputation. Moreover, cilium formation can be modified experimentally, e.g., excessively long cilia are associated with the developmental arrest at the blastula stage, elicited by treatment of early-stage embryos with a variety of agents. This blastula arrest has been generically termed "animalization" (Lallier 1975). In the specific case where zinc ions have been used as the animalizing agent, developmental arrest forming a phenotypically exaggerated blastula may be reversed with the resumption of development (Nemer et

al. 1985a; Nemer 1986). Excessive cilium lengths in variously animalized embryos would seem to reflect altered length restrictions and potentially altered expression of genes for ciliary proteins.

Aspects of cilium formation have been studied by characterizing axoneme regeneration after amputation in sea urchin embryos as well as in unicellular organisms. The major proteins of axonemal microtubules, the α - and β -tubulins, need to be synthesized for complete flagellum regeneration in *Chlamydomonas* (Rosenbaum et al. 1969) and may be drawn from a preexisting pool, as in the case of cilium regeneration in *Tetrahymena* (Guttman and Gorovsky 1979). Deflagellation induces the coordinately increased transcription of the two α - and two β -tubulin genes of *Chlamydomonas* (Brunke et al. 1984; Youngblom et al. 1984; Silflow and Rosenbaum 1985) as well as the stabilization of tubulin mRNA (Baker et al. 1984). In the case of the sea urchin embryo, cilium regeneration could be demonstrated to proceed through an initial phase that is independent of new protein synthesis and a second phase that depends on protein synthesis (Burns 1979). Although the expression of genes for ciliary proteins during the initial phase of regeneration has not been excluded, it was only after repeated rounds of deciliation and regeneration that elevated tubulin synthesis and tubulin mRNA activity assayed in vitro could be detected in sea urchin embryos (Merlino et al. 1978). In spite of a large pool of tubulins

in the egg (Raff et al. 1971; Raff and Kaumeyer 1973), the embryo synthesizes these proteins (Stephens 1972), and accumulates tubulin mRNAs (Alexandraki and Ruderman 1985; Harlow and Nemer 1987) during the developmental period of ciliogenesis. In contrast to the single or duplicate genes for tubulins in *Tetrahymena* (Callahan et al. 1984) and *Chlamydomonas*, the existence of multigene families for tubulins in sea urchins (Alexandraki and Ruderman 1981; Harlow and Nemer 1987) raises the question of whether or not members of these gene families are expressed selectively in association with cilium formation.

The embryos of *S. purpuratus*, express at least four β -tubulin mRNAs (Harlow and Nemer 1987). Three of these, $\beta 1$, $\beta 2$, and $\beta 3$ tubulin mRNAs are present in the egg, begin to increase in amount in the early blastula, and reach peak concentrations in the just-hatched blastula. All three are later enriched in the pluteus ectoderm; however, $\beta 1$ considerably exceeds the other two in this regard. In contrast, $\beta 4$ mRNA, the lowest in abundance, is not detected before the gastrula stage and is highly enriched in the endomesoderm of the pluteus larva. Here we examine the extent to which each of these β -tubulin mRNAs is associated with cilium formation by experimentally altering and/or inducing cilium formation. The coordinate and selective aspects of β -tubulin gene expression we observe in the normal blastula, in the exaggerated blastula phenotype of the zinc-animalized embryo, and in response to deciliation by each of these embryos may provide a basis for determining whether the regulation of these genes bears on the functional diversity of microtubules (Fulton and Simpson 1976) or on the quantitative regulation of tubulin synthesis in specific cell types and circumstances (Raff 1984).

Results

Ciliogenesis in normal and zinc-animalized embryos

To provide a basis for studying the association of β -tubulin gene expression with cilium formation, we compared the quantitative parameters of ciliogenesis in normal and zinc-animalized embryos. Although elongated cilia have been long been noted for zinc-animalized embryos (Lallier 1955, 1975), their lengths have not been measured accurately, as they have been in the case of other animalizing events (Burns 1973, 1979; Riederer-Henderson and Rosenbaum 1979). In *S. purpuratus* the mean length of the cilia, which were isolated and photographed for measurement (Fig. 1A), is 25–35% greater for zinc-animalized embryos (e.g., Fig. 1C) than for normal embryos (e.g., Fig. 1B). The distributions of cilium lengths show that ~10% of cilia from normal embryos are >25 μ m, whereas ~50% of the cilia from animalized embryos are >25 μ m, and the longest cilia of the animalized embryos were longer than the longest in the normal embryo. These distributions are shown diagrammatically in a computer graphics reconstruction of

cilium arrangement (insets of Fig. 1, B and C) in which the cilia are displayed with the longest at the animal pole and the shortest at the vegetal pole, reflecting the apparent size gradient in the photographs of Riederer-Henderson and Rosenbaum (1979). The greater number of long cilia in the zinc-animalized embryo indicates that regulation of cilium length is altered in the zinc-animalized embryo.

To determine whether the longer cilia in the zinc-animalized embryo arose through an enhanced rate, or a sustained duration, of cilium growth, we measured the developmental time course of change in cilium size in normal and animalized embryos (Fig. 1D). Ciliogenesis was readily quantified from the 12th hr postfertilization; however, extrapolation indicated that its initiation occurs between the 8th and the 10th hr (~100-cell stage), consistent with microscopic observations on other sea urchin species (Masuda 1979; Masuda and Sato 1984). The average length of cilia increased at similar rates in the normal and animalized embryos. However, while the rate diminished in the normal embryo so that a plateau value of 19–20 μ m was reached between 20 and 24 hr, cilium elongation in the animalized embryo continued on at the same rate for a longer time and reached a mean length of 25–27 μ m by the 24th hr. Therefore, the greater length of cilia in animalized embryos is attributable to a longer period of sustained growth, rather than to an inherently greater rate of growth of cilia.

Expression of β -tubulin mRNAs in normal and zinc-animalized embryos

The period of ciliogenesis in the normal embryo appears to coincide with a previously defined period of rapid β -tubulin mRNA accumulation in the blastula stages during which three distinct β -tubulin mRNAs, $\beta 1$, $\beta 2$, and $\beta 3$, are present (Harlow and Nemer 1987). To determine whether β -tubulin gene expression is altered in association with the temporal and quantitative changes in ciliogenesis in the zinc-animalized embryo, the amounts of these β -tubulin mRNAs were determined in control and animalized embryos by the reference RNA method (Harlow and Nemer 1987). For illustration, Figure 2 shows the gel blot of one batch of embryos used for these comparisons hybridized successively with an anti-sense $\beta 1$ -3'-specific probe and an anti-sense β -tubulin coding region probe. Known amounts of reference RNA, containing both the $\beta 1$ -3'-specific and coding region probe sequences in a sense orientation were used to determine the molecular equivalents of the β -tubulin RNAs as previously described (Harlow and Nemer 1987). These measurements on four batches of embryos are summarized in Figure 3, which shows a developmental profile of the total amount of β -tubulin mRNA in normal embryos consistent with previous results (Harlow and Nemer 1987a). The concentration of total β -tubulin mRNA in the animalized embryos increases for a longer period and becomes 1.4-fold higher than that of the normal embryos (Fig. 3), in agreement with experiments utilizing a β -tubulin cDNA probe (then identi-

Figure 1. Ciliogenesis in normal and zinc-animalized embryos. (A) Dark-field photograph of cilia isolated from normal embryos (bar represents 20 μ m). (B and C) Length distribution of cilia isolated 24 hr postfertilization from normal and zinc-animalized embryos, respectively. (Insets, B and C) Computer drawings that indicate the distribution of cilium lengths from animal to vegetal poles. The longer cilia are located at the animal pole, which is presented without the excessively long stereocilia. (D) Fertilization envelopes were removed from embryos at 2 hr postfertilization. Normal ($\bullet$) and 500 μ M zinc-animalized (Δ) embryos were cultured to the indicated times at which aliquots of embryos were treated with hypertonic seawater and their cilia collected for analysis.

fied only as Blast[1; Nemer 1986) and exposures long enough to detect only the $\beta 1 + \beta 2$ RNA band, constituting the majority of the β -tubulin mRNAs in these embryos. The maximum concentration of total β -tubulin mRNA occurs 3–4 hr prior to the attainment of the maximum length of cilia in both the normal and animalized embryo. Since the concentration of total β -tubulin mRNA has been correlated with the rate of β -tubulin protein synthesis (Harlow and Nemer 1987), this lag might be attributable to the post-transcriptional events of microtubule assembly in cilia. An increase in the amount of total β -tubulin mRNA above the maternal level is detected after the 8th hr in the control, but not until after the 10th hr in the animalized embryos. Thus, the period of β -tubulin mRNA accumulation is 8 hr (8th to 16th hour postfertilization) in the normal embryo, but $\sim$ 11 hr in the animalized embryos (10th to $\sim$ 21st hour postfertilization). This extended period of total β -tubulin mRNA accumulation coincides

with the period of continued elongation of cilia in the animalized embryo beyond that in the normal embryo.

The schedules of accumulation of the three constituent β -tubulin mRNAs are similar to that of the total in each case; however, the relative proportions of individual species differ in the control and animalized embryos. For the period of 8- to 24-hour postfertilization in the normal embryo the average levels of $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs are represented in the proportions of 0.5:1:0.3. In the counterpart animalized embryo, the average level of $\beta 1$ increased by 60%, while that of $\beta 2$ and $\beta 3$ increased by 20% and 10%, respectively, resulting in a proportionality of 0.7:1:0.3. The higher total β -tubulin mRNA concentration in the animalized embryo is, thus, due mostly to the elevated level of $\beta 1$ mRNA, and the longer period of cilium growth resulting in longer lengths of cilia in the animalized embryos coincides with a sustained elevated concentration of β -tubulin mRNA, especially that of $\beta 1$.

Figure 2. Northern blots of β -tubulin mRNAs from normal and zinc-animalized embryos. Control and zinc-animalized (incubated to 18 hr postfertilization in 400 μ M Zn^{2+}) embryos were cultured to the indicated time postfertilization and RNA extracted from aliquots of embryos. A mixture of total RNA (7 μ g/lane) from these samples and reference RNA (500, 250, 125, 62.5, or 31.25 ng) were electrophoresed and transferred to nitrocellulose. The gel blot was hybridized successively with the ^{32}P -labeled β 1-3' specific probe and then with the ^{32}P -labeled coding region probe as indicated in Materials and methods. Both autoradiographic exposures were for 4 hr.

β -Tubulin mRNAs accumulate during cilium regeneration in normal and zinc-animalized embryos

When embryos that are animalized by treatment of blastulae with trypsin are deciliated through exposure to hypertonic seawater, they regenerate their cilia to their original, characteristically excessive lengths (Burns 1979; Riederer-Henderson and Rosenbaum 1979). Zinc-animalized embryos that have been deciliated regenerate their cilia slightly faster than contemporary, 23-hr normal blastulae (Table 1). The cilia elongated in each case to >70% of the original lengths in the first 2 hr, then continued more slowly, reaching full length between the 6th and 8th hr postdeciliation. Formation of the longest cilia, however could be attributed to continued growth at an undiminished rate beyond the second hour.

The expression of the β -tubulin genes during the course of cilium regeneration was assessed by measuring the levels of the β -tubulin mRNAs over the first 4 hr of regeneration. In both control and animalized embryos, accumulation of the β -tubulin mRNAs was induced for the first 2 hr following a single deciliation at 23 hr postfertilization (Fig. 4). Although the total β -tubulin mRNA concentration in the animalized embryo at this time was considerably greater than in the normal embryo (Fig. 3), the rate of total β -tubulin mRNA accumulation in the animalized embryo was about twice that in the normal

embryo (Fig. 4). By the 2nd hr of cilium regeneration, the animalized embryos had $\sim 5 \times 10^5$ total β -tubulin mRNA molecules per embryo, a level approaching the maximum attained during the developmental period of ciliogenesis (Fig. 3). Subsequently, the concentration declined sharply. In contrast, the accumulation of the total β -tubulin mRNA continued for 4 hr in the normal embryo, approaching the maximum concentration of $\sim 3 \times 10^5$ molecules per embryo attained during ciliogenesis (Fig. 3). The constituent β -tubulin mRNAs accumulated during the first 2 hr of cilium regeneration at the same relative rates in both the normal and animalized embryos. Moreover, in the animalized embryo their amounts declined coordinately after the 2nd hr. Thus, in contrast to the specific enhancement of β 1 mRNA expression during ciliogenesis in the animalized embryo compared to the normal embryo, these β -tubulin mRNAs are expressed in the same proportions (1:1:0.3) during cilium regeneration in the control and animalized embryos. Similar deciliation experiments were performed at the 30-hr stage, in which early gastrulae and their animalized contemporaries contained essentially equivalent β -tubulin mRNA levels, in contrast to the difference in mRNA levels between contemporary normal and animalized blastula stages. The accumulation curves (data not shown) were similar to those of the respective control and animalized embryos in Figure 4, and thus apparently not influenced by the preexisting β -tubulin mRNA level.

Figure 3. Concentration of β -tubulin mRNAs in normal and zinc-animalized embryos. Gel blots of total RNA from normal ($\bullet$) and zinc-animalized (Δ) ($400\text{--}700\ \mu\text{M}\ \text{Zn}^{2+}$) embryos in five developmental series were prepared and probed as shown in Fig. 2. The reference RNA on the same gel blot was used to determine the amounts of $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs, which were summed to obtain the total amount of β -tubulin mRNA. The average values of the total and each RNA are plotted.

Selectively enhanced gene expression during cilium regeneration

Are the levels of $\beta 1$, $\beta 2$, and $\beta 3$ mRNA, induced to increase by a single deciliation, also increased coordinately by repeated rounds of deciliation? Moreover, is the response during cilium regeneration an aspect of a more pervasive stimulation of ectodermal gene expression? To answer the first question, we submitted normal blastulae to three cycles of cilium regeneration, in each of which deciliation by hypertonic seawater was followed by a 2-hr period of regeneration (Fig. 5). The first cycle elicited a 3.1-fold increase in total β -tubulin mRNA; the second and third cycles produced 5.5- and 6.7-fold increases over the controls. Through each cycle the $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs were elevated to similar extents, thus indicating a continued coordination of the expression of these mRNAs. Multiple deciliations of zinc-animalized embryos also resulted in further increases of $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs (data not shown). Under these conditions of pronounced induction of β -tubulin, another gene, Spec

1, whose expression has been localized to the ectoderm (Lynn et al. 1983), and thus operational in cells that are forming cilia, remained essentially unaffected through all three cycles (Fig. 5). Therefore, treatment with hypertonic seawater does not serve as a general stimulant of ectodermal gene activity.

Is the induced expression of other β -tubulin genes excluded during cilium regeneration? Although another β -tubulin mRNA, $\beta 4$, has been shown not to appear until the gastrula stage and to be restricted to the endomesoderm tissue fraction (Harlow and Nemer 1987), its induction during cilium regeneration could not be excluded a priori. The low abundance of $\beta 4$ mRNA necessitated long autoradiographic exposure, which in the case of gastrula RNA resulted in a clear band at 3.0 kb (Fig. 6A). However, this long exposure failed to detect a definitive band of $\beta 4$ mRNA in blastulae even after three cycles of cilium regeneration. Therefore, the $\beta 4$ gene, which is apparently not active in the blastula, cannot be induced by deciliation at this stage. It remained possible, nevertheless, that the expression of $\beta 4$ tubulin mRNA

Table 1. Regeneration of cilia in normal and zinc-animalized embryos

Hours postdeciliation	Mean cilium length in microns (maximum)	
	normal	zinc-animalized
1	8.2 (16)	9.6 (18)
2	16.4 (21)	18.8 (27)
4	19.9 (37)	22.5 (40)
8	22.6 (48)	26.3 (57)

Normal and zinc-animalized embryos, at 22 hr postfertilization, were deciliated and monitored for cilium length as in Fig. 1 during the course of regeneration. The lengths of the longest cilia are also indicated (maximum).

might be induced in response to deciliation only at a developmental stage in which it was already expressed at some detectable level. Therefore, we tested whether $\beta 4$ tubulin mRNA was also excluded from cilium regeneration at the gastrula stage. The level of $\beta 4$ mRNA expressed in gastrulae was unchanged even after two rounds of deciliation (Fig. 6B). Shorter exposures of this same blot revealed, in contrast, that levels of $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs were elevated following the two cycles of deciliation. Therefore, the lack of induction of $\beta 4$ RNA

at a stage when it is being expressed indicates that the $\beta 1$ -, $\beta 2$ -, and $\beta 3$ -tubulin genes are induced selectively in association with cilium formation.

β -Tubulin gene transcription in normal and zinc-animalized embryos and in response to deciliation

Deflagellation of *Chlamydomonas* has been shown to induce tubulin gene transcription directly (Baker et al. 1984; Keller et al. 1984). In the sea urchin embryo a transcriptional response to deciliation was suggested by results of actinomycin D (Merlino et al. 1978). To test this hypothesis, nuclear run-on transcripts from normal and zinc-animalized embryos that had or had not been deciliated were hybridized with a β -tubulin coding region probe to monitor total β -tubulin transcription. The amount of run-on transcripts in nuclei from 23-hr animalized embryos was ~ 3 times higher than that in nuclei of contemporary control blastulae (Fig. 7A). At 1 hr after deciliation the amount in the animalized embryo remained at this high level, while the level in the control blastulae increased to that of the animalized embryo. Deciliation of 28-hr early gastrulae also results in an increase in β -tubulin run-on transcription, whereas metallothionein transcription was unaffected by decilia-

Figure 4. Time course of β -tubulin mRNA concentrations during cilium regeneration in normal and zinc-animalized embryos. Normal and zinc-animalized ($500 \mu\text{M Zn}^{2+}$) embryos, 22 hr postfertilization, were deciliated in hypertonic seawater. A gel blot of total RNA isolated from nondeciliated normal ($\circ$), nondeciliated animalized (Δ), deciliated normal ($\bullet$), and deciliated animalized ($\blacktriangle$) embryos at the indicated times postdeciliation was prepared and probed as shown in Fig. 2. The reference RNA on the same gel blot was used to determine the amounts of $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs, which were summed to obtain the total amount of β -tubulin mRNA.

Figure 5. The selective effect of multiple deciliations in normal embryos. Normal embryos, 24 hr postfertilization, were deciliated three times (1, 2, and 3, respectively) 2 hr apart with hypertonic seawater and allowed to regenerate their cilia for 90 min, at which time samples were taken for RNA extraction both from deciliated embryos and controls, which had not been deciliated. A gel blot of total RNA was prepared and probed as shown in Fig. 2 and the amounts of $\beta 1$, $\beta 2$, and $\beta 3$ determined relative to the reference RNA on the blot. Additionally, the same blot was probed with a ^{32}P -labeled SP6-Spec 1 probe. The exposure times were 3 hr for both tubulin probes and 1 hr for the Spec 1 probe. For each deciliation cycle, the ratio of amount of each RNA in the deciliated sample to its control was calculated and plotted as a bar graph, where (in order from left to right) the bar with diagonal lines represents $\beta 1$, the white bar represents $\beta 2$, the shaded bar represents $\beta 3$, and the black bar represents Spec 1.

tion, but increased with added Zn^{2+} (Fig. 7B). Subsequent examination of 30-hr early gastrulae and their animalized contemporaries also revealed higher β -tubulin run-on transcription in the animalized embryo (Fig. 7C,D). The level in the normal gastrula is elevated approximately threefold as early as 45 min after deciliation and is still at that level at 90 min. Again in the animalized embryo the already high level of transcription is not changed upon deciliation. Therefore, the rate of β -tubulin gene transcription per nucleus in the animalized embryo is higher than in the normal embryo, and moreover remains so during the course of animalized development. In addition, deciliation increases the rate of transcription in the normal embryo compared with that in the animalized embryo. The lack of further increase by deciliation of the animalized embryo suggests that it is already at a maximum rate. Finally, the accumulation

of β -tubulin mRNA during cilium regeneration in the animalized embryo in the face of a constant rate of transcription indicates that its regulation of β -tubulin gene expression is predominantly post-transcriptional, whereas, a transcriptional regulation is evident in the period soon after deciliation in the case of normal embryo.

Figure 6. The expression of $\beta 4$ mRNA in deciliated blastulae and gastrulae. (A) A gel blot of the same RNA samples from control and deciliated embryos described in Fig. 5, along with RNA samples from a gastrula (lane G) control known to have detectable $\beta 4$ RNA, and a blastula (lane B) control known to lack $\beta 4$ RNA was prepared and probed as shown in Fig. 2. The ratio of the amount of $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs in the deciliated sample to its control was similar to that in Fig. 5. The 24-hr exposure shown for the coding region probe detected $\beta 4$ RNA. (B) Total RNA from control (lane C) and deciliated (lane D) normal gastrulae (46 hr postfertilization), deciliated two times 2 hr apart, was extracted 3 hr after the second deciliation. A gel blot was prepared, probed as shown in Fig. 2, and exposed for autoradiography for 2 hr for the $\beta 1$ -3' probe and 2 and 24 hr for the coding region probe.

Discussion

β -Tubulin gene expression associated with the developmental period of ciliogenesis

The first period of regulated tubulin expression in *S. purpuratus* embryos occurs during development through the blastula stages, when microtubules are required prominently for the construction of both mitotic apparatus and cilia. The transition from cleavage to early blastula stages involves a 20-fold increase in the rate of β -tubulin gene transcription (Harlow and Nemer 1987). Beginning at the earliest blastula stage, the $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs accumulate at different rates, but coordinately, each reaching a peak value at the mid-blastula

stage. This accumulation period coincides with a maximum rate of ciliogenesis. Over the next several hours the cilia approach their ultimate lengths at a reduced rate, as the levels of β -tubulin mRNAs decline under post-transcriptional regulation (Harlow and Nemer 1987). Over the entire period, however, the rate of cell division is high but essentially constant (Hinegardner 1967). Thus, while the requirements of both mitosis and cilium formation must be met through a supply of tubulin, changes in β -tubulin mRNA concentration in the blastula stages appear to be more closely correlated with ciliogenesis, since rapid β -tubulin mRNA accumulation coincides with rapid cilium elongation and rapid decline in β -tubulin mRNA concentration, with a declining rate of elongation.

Figure 7. Relative transcription rates of β -tubulin genes in nuclei from control and zinc-animalized embryos at various times post deciliation. Run-on transcripts were elongated with [32 P]UTP in equal numbers of nuclei for each stage as described in Materials and methods. (A) Purified run-on transcripts (5×10^5 Cerenkov cpm) from each reaction of 7×10^7 nuclei isolated from normal and zinc-animalized (see Materials and methods) embryos, 22.5 hr postfertilization, at 0 and 60 min postdeciliation, were hybridized to slot blots of DNA, under probe excess conditions. Aliquots of each reaction were hybridized in separate bags simultaneously to replica slot blots containing pSP64- $\beta 1$ -DNA as a β -tubulin (lanes β -T) probe and pSP64 DNA (lanes B) as a control for nonspecific hybridization. (B) Purified run-on transcripts (1.0×10^6 Cerenkov cpm) from 1.3×10^8 nuclei, isolated from control ($-Zn$) and treated ($+Zn$, 500 μM 25–28 hr) embryos, 28 hr postfertilization, at 0 and 60 min postdeciliation, were hybridized at pSP65-MTa-coding, a metallothionein probe (lanes MT) in addition to the β -tubulin and nonspecific DNA probes. (C) Purified run-on transcripts (1.9×10^6 Cerenkov cpm) from each reaction of 1.1×10^8 nuclei isolated from normal and zinc-animalized (see Materials and methods) embryos, 30.5 hr postfertilization, at 0, 45, and 90 min, postdeciliation, were hybridized to slot blots of DNA. (D) A repeat of the experiment described in C, purified run-on transcripts, 1.5×10^6 Cerenkov cpm, were used. After washing, the slots were exposed for autoradiography for 7 (A), 11 (B), 3 (C), and 12 (D) days.

Coordinate and selective expression of β -tubulin genes associated with cilium formation

Is the expression of β -tubulin genes associated with cilium formation specified by the isotypic protein composition of ciliary microtubules or regulated within a framework of cell-type specificity? To distinguish these possibilities, cilium formation would best be studied in isolation. However, we can only approximate this ideal by experimentally exaggerating the proportion of ciliary microtubule formation. The production of longer cilia during development of the animalized compared to the normal embryo and the specific induction of cilium formation as a result of amputation elicit the synthesis of elevated proportions of ciliary microtubules. The period of ciliogenesis in the animalized embryo is longer than that in the normal embryo and reflects an extended duration of accumulation, as well as attainment of elevated concentrations, of β -tubulin mRNA. In addition, while the accumulations of $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs are temporally coordinated, as they are in the normal embryo, their relative concentrations are different in the animalized embryos, with the most pronounced increase being in $\beta 1$ mRNA. The ratio of $\beta 1/\beta 2$ tubulin mRNA increases from 0.5 during normal ciliogenesis to 0.7 during ciliogenesis in the animalized embryo, and to 1.0 during cilium regeneration in both types of embryo. Thus, an increased proportion of $\beta 1$ mRNA appears to be associated with an increased production of ciliary tubulin. This correlation suggests that ciliary microtubules contain a relatively higher proportion of the $\beta 1$ tubulin isotype than other classes of microtubule. Analogously, axonemal microtubule synthesis in the *Drosophila* testis is apparently supported by selective β -tubulin gene expression (Kemphues et al. 1982). However, while compositional differences have been reported in the tubulins of cilia compared with cytoplasmic structures in sea urchin embryos (Stephens 1978), and functional differences among tubulin isotypes have been proposed (Sullivan and Cleveland 1986; Wang et al. 1986), incorporation of a chimeric chicken–yeast β -tubulin into mouse microtubules (Bond et al. 1986) and the intermingling of even the highly divergent hematopoietic β -tubulin isotype among functionally distinct cytoplasmic microtubules (Lewis et al. 1987) make it unlikely that ciliary microtubules actually require specific tubulin isotypes. The association of $\beta 1$ gene expression with cilium formation might instead be considered a consequence of its highly ectodermal specificity. The 17-fold enrichment of $\beta 1$ mRNA and fivefold enrichment of $\beta 2$ mRNA in the ectoderm relative to the endomesoderm of pluteus larvae result in a $\beta 1/\beta 2$ ratio of 0.7–0.8 in ectoderm and ~ 0.2 in nonectodermal cells at the pluteus stages. Thus, an emphasis on cilium formation tends to be correlated with a shift in the $\beta 1/\beta 2$ ratio toward a value characteristic of differentiated ectoderm.

In addition to mechanisms for regulating the proportions of $\beta 1$, $\beta 2$, and $\beta 3$ mRNA expressed, other mechanisms may trigger the induction of their accumulation and temporally coordinate their sustained expression.

Moreover, other proteins are coordinately synthesized with the tubulins during cilium formation in sea urchin embryos (Stephens 1972, 1977) and flagellum formation in *Chlamydomonas* (Lefebvre et al. 1978, 1980). In the sea urchin embryo one such unidentified protein, termed Spec 3, which is expressed exclusively in the ectoderm of pluteus larvae (Bruskin et al. 1981), is encoded by a mRNA that is expressed in the blastula concurrently with $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs and affected by zinc animalization in the same way as these mRNAs (Nemer 1986). The Spec 3 mRNA recently has been shown to be induced coordinately with β -tubulin mRNA in response to deciliation with hypertonic seawater (Eldon et al. 1987).

Triggering mechanisms have been sought to account for the coordinate expression of the duplicate β - and α -tubulin genes of *Chlamydomonas* induced by deflagellation (Brunke et al. 1984; Youngblom et al. 1984; Siflow and Rosenbaum 1985). However, in this case, as well as for the single tubulin genes of *Tetrahymena* (Callahan et al. 1984), the lack of large multigene tubulin families obviates the question of whether certain tubulin genes may be selectively excluded from induction. In *S. purpuratus*, however, $\beta 4$ -tubulin mRNA is neither expressed during the developmental period of ciliogenesis nor induced by deciliation, not even during the developmental stages when it is normally expressed. The lack of expression in association with the strictly epithelial cell function of cilium formation is consistent with the highly endomesoderm-specific expression of the $\beta 4$ mRNA (Harlow and Nemer 1987). The possibility that the hypertonic seawater treatment served generally to induce genes that are expressed specifically in the ectoderm was dispelled by the failure of the highly ectoderm-specific Spec 1 gene to be induced by this treatment. On the other hand, the question arises that the lack of inducibility of the $\beta 4$ gene in the ectoderm is related to its apparent quiescence in this tissue, or, alternatively to the absence of regulatory elements in this gene that are present in the tubulin genes encoding $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs.

A facultative regulation of the β -tubulin gene family is evident from the display of individualistic developmental profiles and tissue specificities, and from the induced coordinate expression of genes that may otherwise be expressed independently. Within this framework cilium formation would seem to require that the expression of the β -tubulin genes be *trans*-regulated by distinct sets of agents: One set would operate basally to specify the proportions of $\beta 1$, $\beta 2$, and $\beta 3$ tubulins, as well as Spec 3, to be expressed within the framework of ectoderm specificity, which may also encompass the inclusion of Spec 1 and the exclusion of $\beta 4$ tubulin; another set would serve to trigger the selective induction of ciliary proteins.

Altered regulation of β -tubulin mRNA expression in the zinc-animalized embryo and its bearing on cilium formation

The peak concentration of total β -tubulin mRNA in-

duced during cilium regeneration approaches the peak concentration attained during the course of developmental ciliogenesis for both control and animalized embryos. Just as the animalized embryo developmentally produced a higher level of mRNA to form cilia of greater length than the control, the deciliated animalized embryo induced a higher level of mRNA to regenerate cilia of greater length than those regenerated by the control embryo. This enhanced expression reflects a correspondingly higher rate of β -tubulin gene transcription in the animalized embryo. Deciliation induced an increase in the β -tubulin transcription rate per nucleus in the control embryo, bringing it to the level found in the animalized embryo. On the other hand, deciliation of the animalized embryo did not alter its already high rate of β -tubulin gene transcription, which probably represents a maximal rate that cannot be further increased. Thus, rather than being regulated to a lower level as in the normal embryo, transcription of β -tubulin genes in the animalized embryo appears to be in a permanent state of induction, which stems from the original animalizing treatment with zinc during the late cleavage–early blastula stages.

A predominantly post-transcriptional mode of regulation of β -tubulin mRNA in the normal embryo was deduced to operate during the late-blastula period, when the mRNA concentration declined in the face of a constant rate of β -tubulin gene transcription per embryo (Harlow and Nemer 1987). If β -tubulin transcription in the animalized embryo is sustained at a maximum, thus constant, rate in the animalized embryo, then the decay of β -tubulin mRNA from its peak concentration can also be attributable to a predominantly post-transcriptional regulation of transcript stability. The higher peak concentration in the animalized embryo (5×10^5 , compared with 3×10^5 molecules per embryo) indicates that any concentration-dependent feedback control mechanism responsible for such a regulation would be pitched at a different level of sensitivity from that in the control embryo. Such a mechanism, which can be expected to depend directly on the levels of protein generated by the β -tubulin mRNA, has indeed been posited to depend on the pool size of unpolymerized tubulin in mammalian cells (Ben Ze'ev et al. 1979; Cleveland et al. 1981; Caron et al. 1985a), and to involve tubulin mRNA stability in response to microtubule depolymerization in enucleated fibroblasts (Caron et al. 1985b) and during flagellum regeneration in *Chlamydomonas* (Baker et al. 1984). Post-transcriptional regulation is also apparent during cilium regeneration in the animalized embryo, since β -tubulin mRNA levels increase without a change in transcription rate. When the concentration of these mRNAs reach $\sim 5 \times 10^5$ molecules per embryo at only 2 hr postdeciliation, it begins to decline. This concentration may be critical, i.e., relevant to the subsequent decay, since it is the same maximal level attained during development of the animalized embryo. In the case of the deciliated normal embryos, accumulation continues even to the 4th hr postdeciliation, approaching the 3×10^5 level, which, in turn, may be critical for regulation in the

normal embryos. Therefore, animalizing treatment with zinc has not only altered the control of the transcription of the β -tubulin genes ($\beta 1$, $\beta 2$, and $\beta 3$) but has also altered the critical parameters of post-transcriptional control.

Materials and methods

Embryos and embryo RNA

Conditions for culturing embryos of *S. purpuratus* in synthetic seawater (SSW) and extraction of RNA have been described previously (Nemer et al. 1984). The animalization protocol using 450–750 μ M ZnSO_4 has been found to produce similar effects (Nemer 1986). Embryos were deciliated by ~ 20 -sec exposure to hypertonic seawater, SSW adjusted to 1 M NaCl. To allow regeneration of cilia, embryos were resuspended in SSW immediately after this treatment. In certain cases eggs were treated with 0.6% *para*-aminobenzoic acid prior to fertilization and fertilization envelopes removed at 2 hr postfertilization.

Analysis of cilium lengths

During the course of either development or cilium regeneration, the cilium lengths were monitored by releasing the cilia into hypertonic seawater and removing the embryos by centrifugation. The cilia in the supernatant were fixed by the addition of glutaraldehyde to 0.5%, then applied to microscope slides. After air-drying, the slides were immersed in cold 5% trichloroacetic acid and then 95% ethanol. Length measurements were made with a Hipad Digitizer (Houston Instrument Co., Houston) on photomicrographs of the cilia and a stage micrometer, magnified $600\times$. Mean length and length distribution were obtained from measurements on 200–400 cilia for each sample. The long stereocilia ($<1\%$ of the cilia) were not obtained consistently, and thus were eliminated from our calculations and representations.

Representations of embryo cross sections displaying correct lengths and radial distribution of cilia (Fig. 1, B and C insets) were formulated by Dr. Samuel Litwin from an algorithm based on the assumptions that the cilia are approximately uniformly distributed over the surface of a spherical embryo and become monotonically longer from pole to pole, as they appear in the photomicrographs of Riederer-Henderson and Rosenbaum (1979). Tiers of blastomeres were represented by spherical zones of equal height, which by a geometric theorem have equal surface area. Thus, the diameter of a circle, representing the outer surface of the embryo sphere, was divided into a number of parts to represent successive tiers of blastomeres. The number of divisions used accommodated an arbitrary 100 cilia, for comparison purposes only. From each division point a line perpendicular to the diameter was projected to the boundary of the circle. From the point on the circle a radial projection was made equal to the scaled length of the corresponding cilium, taking the cilia in order with the shortest at one pole and the longest at the other. Equal spacing of the division points along a diameter assured that an equal number of cilia would emerge from every equal area zone, i.e., one cilium per blastomere. The outline of these cilia was used to redraw them for spacing at equal angles around the center of the circle.

Synthesis and isolation of RNA probes

Fragments of the $\beta 1$ cDNA, which has been sequenced (Harlow et al. 1988), representing the $\beta 1$ -3' untranslated and $\beta 1$ coding regions were subcloned into SP65 and SP64, respectively, as de-

scribed by Harlow and Nemer (1987a). The *Xba*I–*Taq*I fragment (590 bp) of pSpBc10, previously shown to be indistinguishable from the Spec 1 clones of Bruskin et al. (1981, 1982) by Nemer et al. (1985a) was subcloned into *Xba*I–*Acc*I-digested SP65. *Hind*III-digested pSP65- β 1 3', *Hind*III-digested pSP65-Spec1, and *Eco*RI-digested pSP64- β 1 coding were used to synthesize run-off transcripts as antisense probes with SP6 RNA polymerase, as described in Harlow and Nemer (1987a). The 32 P-labeled probes were purified by acrylamide gel electrophoresis as described.

Synthesis and isolation of the synthetic reference RNA

The reference RNA, a sense strand containing the sequences of the β 1-3'-specific and coding region probes was synthesized as a run-off transcript from *Eco*RI-digested pSP64- β 1-Ref DNA, as described (Harlow and Nemer 1987a), except that in later experiments the [8- 3 H]GTP was diluted to a specific activity of 0.52 Ci/mmol or 10-fold lower than previously used. The resulting 3 H-labeled reference RNA was stable for >5 weeks.

Analysis of RNA levels by the reference RNA method

Procedures for the electrophoresis of RNA and blotting of formaldehyde gels have been described previously (Nemer et al. 1984), except that known amounts of reference RNA, determined accurately from its 3 H specific activity, were added to the sample mixtures prior to heating to 65°C. Conditions for prehybridization, hybridization, and washing of the gel blots have been described previously (Harlow and Nemer 1987a), along with a description of the reference RNA method and the correction of sample RNA input per lane by reference to a mitochondrial transcript. Briefly, the gel blots were probed successively, first with the β 1-3' probe and then the coding region probe. Using appropriate autoradiographic exposures, the number of β 1 RNA molecules was calculated directly by comparing the β 1-3' signal in the 1.8-kb band with that in a known amount of the 0.93-kb reference RNA on the same Northern. The number of β 3 and β 4 RNA molecules was calculated by comparing the coding probe signals in the 2.5-kb (β 3) and the 3.0-kb band (β 4) with the coding probe signal in the reference RNA band. Since β 2 RNA was not resolved from β 1 RNA when probed with the coding probe, β 2 RNA was determined by calculating the total amount of β -tubulin RNA in the 1.8-kb band as detected by the coding region probe and subtracting the amount of β 1 RNA determined using the β 1-3'-specific probe. The amounts of the four β -tubulin RNAs could be converted directly into the number of molecules per embryo, since the total amount of RNA per embryo (3.0 ± 0.2 ng) is essentially constant throughout development (Nemer et al. 1984).

Nuclear run-on transcription

Nuclei were isolated from control and treated embryos as described by Marzluff and Huang (1984). The purified nuclear suspension, containing the indicated number of nuclei from each preparation, was incubated in a volume of 0.2 ml at 22°C for 45 min under a single set of conditions, previously described (Wilkinson and Nemer 1987). Equal amounts of radioactivity of the recovered nuclear RNA were hybridized separately to an excess amount (5 μ g) of linearized pSP64- β 1-Ref DNA, as a β -tubulin probe and linearized pSP64 DNA, as a nonspecific probe, immobilized on nitrocellulose as slot blots. In some experiments metallothionein transcription was also monitored by utilizing pSP65-MTa-Coding, which contains the 220-bp *Pst*I–*A*/uI fragment encompassing the coding region of MTa cDNA (Nemer et

al. 1985a) subcloned into the *Pst*I–*Sma*I sites of SP65. Following hybridization, the filters were washed and treated with 20 μ g/ml of RNase A as previously described (Harlow and Nemer 1987a), and finally rewashed in $0.3 \times$ SSC at 60°C, and subjected to autoradiography.

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