Developmental and tissue-specific regulation of β -tubulin gene expression in the embryo of the sea urchin *Strongylocentrotus purpuratus*

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Four β -tubulin mRNAs in the embryo of the sea urchin *Strongylocentrotus purpuratus* are transcribed from at least 3 of the 9–12 β -tubulin genes. A β 1 tubulin mRNA of 1.8 kb, transcribed from a unique β 1 gene, is expressed with high specificity in the pluteus ectoderm. Another 1.8-kb mRNA, β 2, and a 2.5-kb β 3 mRNA are moderately ectoderm specific. In contrast, a 3.0-kb β 4 mRNA is highly specific for the endomesoderm tissue fraction. Certain similarities in developmental and tissue-specific expression suggest that these β -tubulin genes may be related in their mode of regulation to counterparts among the genes for actin, another cytoskeletal protein. Measurements of absolute amounts revealed a distinct developmental profile for each β -tubulin mRNA. An increase in the total amount of β -tubulin mRNA in the early blastula was correlated with an increase in transcription rate per nucleus; whereas, later in the mesenchyme blastula stage, the β -tubulin mRNA level decreased sharply as the rate of β -tubulin gene transcription on a per embryo basis remained constant. Thus, during development through the blastula stages, there was a switch to a predominantly posttranscriptional regulation of β -tubulin mRNA expression, probably through a decrease in mRNA stability.

[Key Words: β -Tubulin mRNAs; sea urchin embryo; tissue specificity; developmental gene expression; transcription rate; quantification method]

Received November 17, 1986; revised version received and accepted December 18, 1986.

Tubulins, the major proteins of microtubules, are the basis for the construction of mitotic spindles, cilia, flagella, and cell-shape determinants. The primary sequences of both α - and β -tubulins are highly conserved (Alexandraki and Ruderman 1983; Cleveland and Sullivan 1985; Harlow and Nemer, in prep.), nevertheless, individual tubulin genes in many cases are expressed differentially (Burland et al. 1984; Natzle and McCarthy 1984; Alexandraki and Ruderman 1985). The expression of functionally different polypeptides is suggested by the identification of conserved variable-region sequences that seem to define four distinct isotypes (Sullivan and Cleveland 1986). However, there is no example of an exclusive cell type specificity for any α - or β -tubulin gene (Cleveland and Sullivan 1985), except in the case of a divergent erythrocyte β -tubulin (Murphy and Wallis 1983). Thus, it has been difficult to demonstrate clear-cut associations between individual tubulins and specific cellular functions. In addition, such diversity of gene function is excluded in organisms that contain a single α - or β -tubulin gene (Callahan et al. 1984) or identical multiples of these genes (Brunke et al. 1984).

The expression of tubulin multigene families (Cleveland et al. 1980; Alexandraki and Ruderman 1981) may be studied advantageously in the embryogenesis of sea

urchins, whose various aspects of development are known in great detail (Czihak 1975; Davidson 1986; Giudice 1986). Several tubulin mRNAs are present in the eggs of the species *Lytechinus pictus* and the expression of tubulin mRNAs is developmentally regulated, as evidenced by increases in their levels during development to the blastula stage (Alexandraki and Ruderman 1985). These increases are correlated with an overall increase in translatable tubulin mRNA (Infante and Heilman 1981; Alexandraki and Ruderman 1985) and an increase in tubulin protein synthesis (Harkey and Whiteley 1983). Cell-type changes become evident later in the gastrula, where tubulins are preferentially synthesized in the epithelial cells, but not in the primary mesenchyme cells (Harkey and Whiteley 1982). Synthesis of tubulin restricted in this manner has not been previously related to the expression of specific tubulin genes in the embryo.

Morphogenesis of the sea urchin blastula requires increased microtubule synthesis for the formation of mitotic spindles and cilia. To analyze how tubulin gene expression is regulated and quantitatively related to these processes in embryogenesis, we have set out to characterize the tubulin gene transcripts expressed during this and subsequent periods of development. In later embry-

Figure 1. The β -tubulin genes detected on gel blots of genomic DNA restriction fragments by probes derived from the $\beta 1$ cDNA. (A) A partial restriction map of the $\beta 1$ cDNA is shown with its 880-bp coding region (open box) and 320 bp of 3' untranslated region (line) and the locations of the coding-region probe and the $\beta 1$ 3'-specific probe fragment. Restriction sites are: (B) *Bgl*II; (S) *Sac*I; (T) *Taq*I; and (E) *Eco*RI. (B) The $\beta 1$ 3'-probe was hybridized with the sperm DNA of two individuals digested with *Hind*III (lanes 1 and 2) or *Eco*RI (lanes 3 and 4) at high stringency. The coding-region probe was hybridized with a replica blot and washed at moderate stringency (C) and later at high stringency (D).

onic stages, tubulin in microtubules and actin in microfilaments are among the cytoskeletal proteins of differentiated cells. The expression of members of the actin gene family in *Strongylocentrotus purpuratus* has been shown to be cell-type specifically regulated (Cox et al. 1986). Hence, we have asked whether the β -tubulin genes might also be expressed under tissue-specific regulation, and thus are potentially coordinated with the expression of genes encoding other cytoskeletal proteins. To perform these analyses, we have made use of a β -tubulin cDNA (Sp- $\beta 1$) of *S. purpuratus* (Harlow and Nemer, in prep.) to construct specific and general β -tubulin probes for detecting and quantifying the β -tubulin genes and their transcripts, and to develop an accurate

gel blot method for measuring absolute quantities of specific RNA transcripts.

Results and discussion

The β -tubulin gene family of *S. purpuratus*

Fragments of $\beta 1$ cDNA as probes to distinguish β -tubulin genes The many examples of 3' untranslated regions serving as gene-specific probes (Bond et al. 1984; Lee et al. 1984; Elliot et al. 1985; Lewis et al. 1985a,b) led us to construct a Sp- $\beta 1$ -specific probe from the *Eco*RI–*Taq*I fragment in the 3' untranslated region of the $\beta 1$ cDNA (Fig. 1A). In contradistinction, all of the β -tubulin genes could be detected by a probe for the

coding region (Cleveland et al. 1980; Alexandraki and Ruderman 1981). Although α -tubulin and β -tubulin gene sequences generally fail to cross-hybridize in other systems (Cleveland et al. 1980; Alexandraki and Ruderman 1981), we, nevertheless, minimized high local homology by choosing the *SacI*–*SacI* fragment as a β -tubulin coding-region probe. Its homolog in the $\beta 3$ tubulin of *L. pictus*, when compared with α -tubulin sequences of the same species, showed only 8% amino acid homology, and less than 35% nucleotide homology, much below the minimum of 50–55% homology that will allow cross-hybridization even at low stringency (Jones et al. 1979; Sim et al. 1979; Jones and Kafatos 1982). The probe derived from the *EcoRI*–*TaqI* fragment in the 3' untranslated region of Sp- $\beta 1$ is designated the $\beta 1$ 3'-specific probe, while the probe derived from the *SacI*–*SacI* fragment of the β -tubulin coding-region is designated the coding-region probe.

A single $\beta 1$ -tubulin gene exists in the β -tubulin multi-gene family To characterize the $\beta 1$ -tubulin genes by restriction fragment analysis, the genomic DNA from each of several individuals was digested with either *HindIII* or *EcoRI*, gel-blotted, and hybridized with the $\beta 1$ 3'-specific probe. In all cases, two of which are shown in Figure 1B, at most two bands hybridized. Since the genome of *S. purpuratus* is highly polymorphic (Britten et al. 1978) and probes for unique genes usually react with two different restriction fragment alleles in any individual sperm DNA preparation (Thomas et al. 1982; Posakony et al. 1983; Lee et al. 1984), our results suggest the presence of a unique $\beta 1$ tubulin gene. In contrast, hybridization of the coding-region probe with the genomic DNAs of these same individuals revealed up to 20 bands with extensive polymorphism (Fig. 1C). Cleveland et al. (1980) similarly detected about 20 bands by probing *HindIII*-digested *S. purpuratus* DNA with a chicken β -tubulin cDNA. These results, obtained with either the heterologous or the homologous coding probe, suggest about 10 β -tubulin genes in this species. All but three to six of the multiple bands detected by hybridization with the coding-region probe can be eliminated by increasing the stringency of washing (Fig. 1D). Of those remaining, one to two correspond to $\beta 1$; hence two to four bands represent two genes different from $\beta 1$ but having a high degree of homology to $\beta 1$ in this part of the coding region.

To determine the precise number of $\beta 1$ genes, as well as the number of genes in the β -tubulin family, the same two probes were hybridized with known amounts of genomic DNA on slot blots relative to standards consisting of varying amounts of a plasmid DNA, containing both probe sequences. Densitometric analysis of two individuals indicated 0.8 and 0.9 copies of the $\beta 1$ gene per haploid genome (Fig. 2A). Therefore, a single $\beta 1$ gene is present in the *S. purpuratus* genome. In contrast, there are a total of about 11 β -tubulin genes per haploid genome, since hybridization with the coding-region probe indicated 9 and 12 genes for these same individuals (Fig. 2B). A similar range (11–13) has been esti-

mated for the β -tubulin family of another sea urchin, *L. pictus* (Alexandraki and Ruderman 1981). When the blot hybridized with the coding-region probe was washed using high stringency conditions (Fig. 2C), a total of three to four genes remained. Since there is one $\beta 1$ gene per haploid genome, two to three other genes are $\beta 1$ -like in that they resemble the $\beta 1$ gene in this part of the coding region more closely than the other β -tubulin genes; however, since at high stringency the $\beta 1$ 3' probe does not hybridize to these genes, they are distinct from $\beta 1$. These genes plus $\beta 1$ constitute a subclass of β -tubulin genes.

Characterization of β -tubulin mRNAs expressed in embryos

Detecting and distinguishing individual β -tubulin mRNAs To characterize the β -tubulin gene transcripts, we successively hybridized the $\beta 1$ 3'-specific probe and the coding probe to gel blots of total embryo RNA from two developmental stages. The $\beta 1$ 3'-specific probe hybridized with a single 1.8-kb RNA band in both the blastula and pluteus stages (Fig. 3). The coding-region probe, however, detected bands of 1.8 and 2.5 kb in RNA from blastulae, and these bands together with a third band of 3.0 kb in RNA from pluteus larvae. Long autoradiographic exposures showed no hybridization of

Figure 2. Estimation of β -tubulin gene number in *S. purpuratus* genomic DNA. Nitrocellulose slot blots were prepared by loading two different genomic DNAs as samples and the indicated haploid genome equivalents of linearized pSP64- $\beta 1$ -Ref DNA as standards. (A) One blot was hybridized with the $\beta 1$ 3'-specific probe and washed at high stringency. On this blot sample rows 1 and 3 contain 1 μ g, while rows 2 and 4 contain 3 μ g of *PstI*-digested genomic DNA. (B) Another blot was hybridized with the coding-region probe and washed at moderate stringency. On this blot rows 1 and 3 contain 0.5 μ g, while rows 2 and 4 contain 1 μ g of *PstI*-digested genomic DNA. (C) The blot from B was washed at high stringency.

Figure 3. Detection of distinct β -tubulin mRNAs on a gel blot of total RNA from early- and late-stage embryos. A mixture of total RNA (7 μ g/lane) from blastulae and plutei and 250 pg and 62.5 pg, respectively, of 3 H-labeled reference RNA, were electrophoresed and transferred to nitrocellulose. The gel blot was hybridized successively with the β 1 3'-specific probe (lane 1) and then with the coding-region probe (lanes 2–4) as described in Materials and methods. Lanes 1 and 2 are 4-hr exposures, while lanes 3 and 4 are 12- and 60-hr exposures, respectively. Illustrated below is a partial restriction map of β 1 cDNA and the locations of the sense-strand reference RNA and the anti-sense coding and 3' probes.

β 1 3'-specific probe with the 2.5- or 3.0-kb RNAs (data not shown).

We next asked whether the β 1 mRNA accounted for all or only part of the RNA in the 1.8-kb band. If the 1.8-kb band consisted only of β 1 mRNA, then the probes from the coding and β 1 3'-specific regions of this mRNA would hybridize to this band in equimolar proportions. If other species were present together with β 1 mRNA, then an additional amount of hybridization of the coding-region probe would reflect the amount of the other species. These alternatives can be distinguished by measuring the successive hybridization of the two probes with the sample RNA relative to a reference containing both the probed sequences, β 1 3'-specific and coding, in a (necessarily) 1 : 1 molar ratio. For this purpose, a 931-base sense-strand RNA was transcribed in vitro from the *Bgl*II–*Eco*RI fragment of β 1 cDNA subcloned into SP64 (Fig. 3). Accurately determined amounts of this reference RNA were mixed with RNA samples, gel electrophoresed, and transferred to nitrocellulose. After the gel blot was hybridized with the β 1 3'-specific probe, the signal in the 1.8-kb band was compared with the signal for the reference RNA, which migrates well ahead of the β -tubulin bands, to indicate the molecular equivalent of β 1 mRNA. After removal of the β 1 3'-specific probe, a similar analysis was performed with the coding-region probe on the same gel blot. Densitometry showed that the molecular equivalent of the coding-region probe hybridized to the 1.8-kb band, relative to the reference RNA, was greater than three- and greater than twofold more than the molecular equivalent

of the β 1 3' probe hybridized to this band in the blastula and pluteus stages, respectively (Fig. 3, lanes 1 and 2). Therefore, the 1.8-kb band from both developmental stages contains β 1 RNA plus additional non- β 1 tubulin mRNA, which we shall designate β 2. Whether or not β 2 is a single species remains to be determined. Therefore, in the *S. purpuratus* pluteus there are at least four β -tubulin mRNAs: β 1 (1.8 kb), β 2 (1.8 kb), β 3 (2.5 kb), and β 4 (3.0 kb). These mRNAs are almost entirely poly(A)⁺ RNA, since greater than 80% could be bound to oligo(dT)-cellulose and the hybridization signals in these bands from both probes resisted treatment with ribonuclease A to the same extent (data not shown).

Distinguishing β -tubulin mRNAs as transcripts of different genes The β 1 mRNA is clearly distinguished from the other β -tubulin mRNAs by virtue of its hybridization with a specific probe from its 3' untranslated sequence. However, it cannot be concluded, strictly on the basis of the differences in their sizes, that the other β -tubulin mRNAs are transcribed from different genes. To test this proposition, we examined their degrees of homology to the β 1 coding region, as indicated by differences in melting temperatures of hybrids formed between each mRNA and the coding-region probe derived from β 1. The rationale was that mRNAs transcribed from the same gene would have identical nucleotide sequences in the coding region and that discrepancies between T_m values could arise only if the mRNAs were transcribed from different genes. The coding-region probe was annealed to total pluteus RNA and reference

Figure 4. Melting profiles of individual β -tubulin RNAs. A mixture of pluteus RNA (7 $\mu\text{g}/\text{lane}$) and ^3H -labeled reference RNA (62.5 pg/lane) was applied to 30 lanes and electrophoresed as in Fig. 3. The gel blots were cut into individual strips for hybridization with the coding-region probe and subsequently washed in $0.1 \times \text{SSC}$ at the indicated temperatures. (A) Representative autoradiograms of these strips are shown, in which lanes 1–6 represent temperatures of 65°, 70°, 75°, 79°, 83°, and 85°C. The amounts of the individual RNAs were determined by densitometer tracing, directly in B for the $\beta 3$ (○) and $\beta 4$ (△) RNAs. C (●) the melting profile of the 1.8-kb ($\beta 1 + \beta 2$) band (●) was compared with that of the reference RNA (X). Since the melting profile of the reference RNA represents that of $\beta 1$ RNA, the melting profile of $\beta 2$ RNA (— — —) was obtained by subtracting that of the reference RNA ($\beta 1$) from that of the 1.8-kb ($\beta 1 + \beta 2$) band.

RNA on gel blots, then the probe was melted off at increasing temperatures (Fig. 4). The melting profile of the reference RNA, representing $\beta 1$ mRNA, was subtracted from the profile of the 1.8-kb RNA ($\beta 1$ plus $\beta 2$) to give that of $\beta 2$ mRNA. The melting profiles of the 2.5-kb $\beta 3$ and 3.0-kb $\beta 4$ mRNAs were obtained directly. The T_m values of the RNA:RNA duplexes are $\beta 1$ - 80.5°, $\beta 2$ - 74.3°, $\beta 3$ - 74.6°, and $\beta 4$ - 73.0°C. The T_m values of $\beta 1$ and $\beta 4$ mRNAs are different from each other and from the others. However, the difference for $\beta 2$ and $\beta 3$ mRNAs is not large enough to merit a conclusion concerning their genic origins; although their T_m values are similar, they could have different sequences and be transcripts from different genes. Alternatively, $\beta 2$ and $\beta 3$ mRNAs may be derived from the same gene as the result of alternative polyadenylation sites, similar to the 1.8- and 2.6-kb RNAs derived from the human M40 β -tubulin gene (Lee et al. 1983). Until further studies distinguish between these alternatives, the differences in T_m value indicate that at least three different genes are

transcribed during the embryonic development of *S. purpuratus*.

Developmental and tissue-specific regulation of β -tubulin gene expression

Quantification of β -tubulin mRNAs The measurement of the molecular equivalents of the $\beta 1$ 3'-specific and coding-region sequences by hybridizing each with its respective probe relative to the reference RNA can be used to calculate not only the amounts of the 1.8-kb $\beta 1$ and $\beta 2$ mRNAs, as described above, but also the amounts of the 2.5-kb $\beta 3$ and 3.0-kb $\beta 4$ mRNAs. The wide range of abundances of the β -tubulin mRNAs was accommodated by varying the amount of reference RNA and the time of autoradiographic exposure. The assay had a standard deviation of 15%. We established that this "reference RNA method" was applicable over a useful abundance range by virtue of the linearity of hybridization signal for each probe in the concentration

Figure 5. Measurement of the amount of β -tubulin RNA by the reference RNA method (A, B, and C) and the solution hybridization method (D). Mixtures of sample RNA and serial dilutions of reference RNA were electrophoresed, blotted and hybridized successively with the $\beta 1$ 3'-specific (A, $\bullet$) and the coding region (B, X) probes as in Fig. 3. Two different blastula samples are shown with their reference RNA bands, which correspond to the first two lanes of the dilution series of reference RNA. (C) The autoradiographs were scanned by densitometry and a standard curve was plotted as hybridization signal for each probe versus the amount of input reference RNA. (D) For measurement by solution hybridization, tracer-excess titrations of the β -tubulin RNA in the total RNA from 20-hr swimming blastulae were performed using the $\beta 1$ 3'-specific ($\bullet$) or coding-region probe (x). After hybridization, the quantity of RNA-RNA duplex was assayed as described in Materials and methods.

range of the β -tubulin mRNAs in the embryo samples tested. As an example, gel blots are shown for two blastula samples with a series of different amounts of the reference RNA run on the same gel, hybridized successively to the $\beta 1$ 3'-specific probe (Fig. 5A) and to the coding-region probe (Fig. 5B). By densitometry we determined that both signals were linear in this range (Fig. 5C).

The accuracy of this reference RNA method was tested by comparison with the solution hybridization method as described by Lee et al. (1986). Blastula RNA was hybridized in solution with excess amounts of either probe. Using the slopes derived from plots of the

RNase-resistant counts per minute versus the total RNA input (Fig. 5D), and the specific activities of the $\beta 1$ 3'-specific and coding probes (813 and 645 disintegrations/minute per picogram, respectively, in this experiment), we calculated that this blastula sample contained 1.18×10^5 molecules of $\beta 1$ mRNA/embryo and a total of 2.50×10^5 β -tubulin mRNA molecules/embryo. Without gene-specific probes, the only additional conclusion we can draw from these measurements, based on solution hybridization procedures, is that the blastula sample contains, besides the $\beta 1$ mRNA, an additional 1.32×10^5 molecules of β -tubulin mRNA/embryo. However, using the reference RNA method (Fig. 5A,B)

we determined that this blastula sample contained 1.07×10^5 , 1.98×10^5 , and 0.21×10^5 molecules/embryo of $\beta 1$, $\beta 2$, and $\beta 3$ mRNAs, respectively, or a total of 3.26×10^5 β -tubulin mRNA molecules/embryo. The excellent agreement of the $\beta 1$ and total β -tubulin mRNA values between the two methods indicates that the reference RNA method is at least as accurate as the solution hybridization method.

Concentration of individual β -tubulin mRNAs in eggs and embryos at various stages of development
The relative amounts of the individual β -tubulin mRNAs in eggs and embryos at different stages are exemplified by the two different developmental series shown in Figure 6. No increase in amount of the β -tubulin mRNAs is apparent before 9 hr; however, their increase in size between 0 and 6 hr can be correlated with polyadenylation, since the percentage of β -tubulin RNA bound to oligo(dT)-cellulose increased (unpublished data). Accumulation is detected between 9–12 hr postfertilization (morula to early blastula stage). The amounts in the 1.8-kb ($\beta 1$ plus $\beta 2$) band and the 2.5-kb $\beta 3$ band increase substantially in development from egg to blastula stage. The $\beta 3$ mRNA constitutes a larger proportion of the total at the pluteus stage than at earlier stages. In contrast, $\beta 4$ mRNA is not detected until the gastrula stage, and even then requires a long autoradiographic exposure.

Utilizing the reference RNA method, we measured the amounts of the individual β -tubulin mRNAs in samples of developmental series from six different batches of embryos. The multiple values for each time point were averaged to yield the values plotted in Figure 7. Each β -tubulin mRNA displays a distinct developmental profile. The $\beta 1$ and $\beta 2$ mRNAs constitute 96%

of the maternal β -tubulin mRNA; both are present at 0.13×10^5 molecules per egg, while $\beta 3$ mRNA is at one-tenth this value. Accumulation of $\beta 1$ mRNA begins during the morula–blastula transition (9–10 hr) and reaches a maximum value of 1.1×10^5 molecules/embryo at the swimming blastula stage. Thereafter, the amount of this mRNA decreases by about half during development to the gastrula, then increases during development from gastrula to pluteus, again reaching 1.0×10^5 molecules/embryo. Although $\beta 2$ mRNA follows a similar pattern, its peak is slightly earlier at the rotating blastula stage where it reaches a higher value (2.0×10^5 molecules/embryo) than $\beta 1$ mRNA. By the gastrula stage the amount of $\beta 2$ mRNA has decreased to one-fifth of its maximum, becoming similar in amount to $\beta 1$ mRNA. Later, it increases to 1.7×10^5 molecules/embryo in the pluteus. The developmental profile of $\beta 3$ mRNA resembles that of $\beta 1$ and $\beta 2$ mRNAs in the early-stage embryo, but its levels are significantly lower, reaching only 0.5×10^5 molecules/embryo in the rotating blastula. In contrast to $\beta 1$ and $\beta 2$ mRNAs, which attain their maximum levels in the blastula, the maximum level of $\beta 3$ mRNA (1.2×10^5 molecules/embryo) is in the pluteus. Originally at less than 5% of total β -tubulin mRNA in the egg, $\beta 3$ mRNA is 30% of the total at this stage. Even more restricted during development than the other β -tubulin mRNAs, $\beta 4$ mRNA cannot be detected until the late gastrula stage, after which in the pluteus it reaches a level of 0.11×10^5 molecules/embryo (less than 5% of total β -tubulin mRNA). The profile of total β -tubulin mRNAs displays successive peaks at the blastula and pluteus stages, with an intervening minimum during the gastrula stages (Fig. 7). A similar profile for total β -tubulin protein synthesis in *L. pictus* was observed by Alexandraki and Ruderman

Figure 6. Characterization of β -tubulin RNAs at different embryonic stages by gel-blot hybridization. Gel blots of total RNA (7 μ g/lane) of embryos in a series of developmental stages from separate batches of eggs (right and left panels) were hybridized successively first with the $\beta 1$ 3'-specific probe, then with the coding-region probe as in Fig. 3. Exposures with the $\beta 1$ 3'-specific and the coding probe were 2 hr for the blots on the left and 4 hr for the blots on the right. Longer exposures are presented of the blots with the coding probe: 4 hr on the left to show the egg and cleavage stages and 44 hr on the right to show the $\beta 4$ RNA.

(1981). Their subsequent description (1985) of two distinct β -tubulin mRNAs also revealed increases from the egg to blastula stage, but did not correspond in later stages to the profiles we have observed with *S. purpuratus*.

Figure 7. Quantitative changes of β -tubulin mRNAs during development. Developmental profiles of $\beta 1$ ($\bullet$), $\beta 2$ ($\times$), $\beta 3$ ($\circ$), and $\beta 4$ ($\triangle$) RNAs, were obtained by determining the amount of these RNAs in samples from six different developmental series using the reference RNA run on the same Northern as a standard as in Figs. 3 and 5. The amounts of the four RNAs were summed to obtain the total amount of β -tubulin RNA ($\square$).

Rates of β -tubulin synthesis correlated with β -tubulin mRNA concentrations The rate of β -tubulin protein synthesis also increases substantially during development. From the rates of total protein synthesis in *S. purpuratus* of 250 and 550 pg/hr at, respectively, the cleavage and blastula stages (Goustin and Wilt 1981), and total microtubule protein synthesis being 0.4% and 1% of total protein synthesis at these respective stages, measured by Raff and Kaumeyer (1973) in *Arbacia punctulata*, we estimate that the rate of β -tubulin protein synthesis increases from 0.5 pg/hr at cleavage to 2.8 pg/hr in the blastula. Since the 1335 bases of the β -tubulin-coding region should contain about 10 ribosomes (Brandis and Raff 1978; Whiteley and Mizuno 1981) with an elongation rate of 1.8 amino acids/sec per ribosome (Goustin and Wilt 1981; Martin and Miller 1983), the 0.3×10^5 β -tubulin mRNA molecules/embryo in late cleavage would synthesize about 0.4 pg of β -tubulin protein/hr per embryo, while the 3×10^5 β -tubulin mRNA molecules in the hatched blastula would synthesize about 3.6 pg of β -tubulin protein/hr per embryo. The reasonable agreement in these respective rates, based on independent measurements and assumptions, suggests that all β -tubulin mRNAs are fully engaged in protein synthesis at the stages under consideration. This agreement and the observation that tubulin mRNAs are associated entirely with polysomes at these stages in *L. pictus* (Alexandraki and Ruderman 1985) support the conclusion that the developmental changes in β -tubulin mRNA levels reflect changes in β -tubulin synthesis.

Transcription rates during early developmental stages To test whether the initial increase in β -tubulin mRNA concentration is regulated at the level of transcription, we examined run-on transcripts from nuclei prepared at each of three early embryonic stages: 6-hr cleavage, 12-hr early blastula, and 23-hr mesenchyme blastula (Fig. 8). The in vitro-labeled RNA was hybridized to SP64- $\beta 1$ -Ref DNA, which comprises 75% as $\beta 1$ coding region and 25% as $\beta 1$ 3' untranslated sequence, and thus represented total β -tubulin transcription. The inclusion of heparin during the labeling of nuclei allowed primarily the elongation of transcripts (Groudine et al. 1981), thus reflecting either the number of RNA polymerase molecules specifically involved or the rate of transcription. In contrast to histone gene transcription, which was substantial in nuclei from the cleavage-stage embryos and considerably diminished in the 12-hr blastula (data not shown), the rate of β -tubulin gene transcription per nucleus relative to the 6-hr cleavage stage increased 20-fold between 6 and 12 hr, and then declined by the 23rd hr to 40% of the 12-hr value. It is not known whether this average decline per nucleus, associated with an increase in cell number from 200 to 500 per embryo, reflects an overall decrease in nuclear rate or unequal rates for different cells of the mesenchyme blastula. In either case, the β -tubulin transcription rate per embryo (rate per nucleus times cell number per embryo) does not change during subsequent development through the 12- to 23-hr blastula stages. Consequently, the overall contribution of newly transcribed β -tubulin

RNA must be taken as constant when evaluating changes in β -tubulin mRNA metabolism during the blastula stages.

A developmental shift in mode of regulation The striking accumulation of total β -tubulin mRNA in the 10- to 16-hr early blastula contrasts with the subsequent, sharp decrease in the 20- to 25-hr mesenchyme blastula (Fig. 7). The increased rate of β -tubulin gene transcription during the cleavage-early blastula transition indicates that the early-stage accumulation of β -tubulin mRNA is transcriptionally regulated. However, the constancy of the overall β -tubulin transcription rate during the subsequent early and late blastula stages indicates that the decline in concentration of β -tubulin mRNA in the mesenchyme blastula cannot be attributed predominantly to a change in the rate of synthesis, but to post-transcriptional events. We conclude that there is a developmental shift in the predominant mode of regulation from transcriptional to post-transcriptional.

The decline of total β -tubulin mRNA in the mesenchyme blastula can be caused by a change in RNA processing and/or degradation. By examining two extreme possibilities we can suggest limits for the stability of β -tubulin mRNA in the late blastula. If there is a sudden failure of processing to yield new mRNA molecules, then the net loss of total β -tubulin mRNA in the mesenchyme blastula would reflect in its slope of decay (Fig. 7) a $t_{1/2}$ of 5 hr for the preexisting mRNA molecules, a value similar to that of the average mRNA in the embryo (Nemer et al. 1975; Galau et al. 1977). On the other hand, if all β -tubulin transcripts are processed and the β -tubulin mRNAs are stable during the early blastula period, then their accumulation rate of 0.64×10^5 molecules/hr per embryo (Fig. 7) can be taken as their synthetic rate. Since the total β -tubulin transcription rate is the same in the early blastula and mesenchyme blastula, this synthetic rate and the rate of loss of 0.34×10^5 molecules/hr per embryo in the mesenchyme blastula can be used to calculate a decay rate of 0.98×10^5 molecules/hr per embryo and a $t_{1/2}$ of 70 min for β -tubulin mRNA at this stage. The shift in the regulation of total β -tubulin mRNA expression during development through the blastula stages may involve a change from negligible turnover of these mRNAs to a $t_{1/2}$ having a minimum value of 1 hr and a maximum of 5 hr.

Changes in stability of tubulin mRNAs have been demonstrated in several systems where tubulin synthesis is modulated, e.g., following deflagellation of *Chlamydomonas reinhardtii* (Baker et al. 1984), the cell cycle in *Physarum* (Schedl et al. 1984), and the differentiation of a teratocarcinoma cell line (Howe et al. 1984). Furthermore, the destabilization of tubulin mRNAs has been implicated in the decrease in tubulin synthesis, correlated with an enlarged pool of depolymerized subunits in colchicine-treated, tissue culture cells (Cleveland and Havercroft 1983; Caron et al. 1985). Since the peak concentration of β -tubulin mRNA in the sea urchin blastula occurs at the end of the major period of rapid cell division and ciliogenesis, an overshoot in β -tu-

bulin synthesis with the accumulation of unpolymerized subunits is possible. An autoregulatory mechanism involving tubulin mRNA stability may thus be operative not only in colchicine-treated cells, but also normally in the late-stage sea urchin blastula.

Tissue specificity of the β -tubulin mRNAs We determined the distribution of the four β -tubulin mRNAs in pluteus larvae, utilizing the tissue fractionation procedure of McClay and Chambers (1978) and the reference RNA method. From four experiments, of which Figure 9 is an example, we calculated for each β -tubulin mRNA mean values of molecules per unit RNA mass in each tissue fraction. The ratio of these values was taken as the specific enrichment for each mRNA. The $\beta 1$ mRNA is highly ectoderm specific, since its amount in this tissue fraction is 17-fold higher than in the endomesoderm fraction. The $\beta 2$ and $\beta 3$ mRNAs are also enriched in the ectoderm, but only about five- and threefold, re-

Figure 8. Relative transcription rates of the β -tubulin genes in nuclei from 6-hr morula, 12-hr early blastula, and 23-hr mesenchyme blastula. Run-on transcripts were elongated with [32 P]UTP in equal numbers of nuclei for each stage as described in the Materials and methods. Purified run-on transcripts, 7×10^5 Cerenkov cpm from each reaction, representing 2.7×10^7 , 1.4×10^7 , and 1.1×10^7 nuclei from 6-, 12-, and 23-hr embryos, respectively, were hybridized to separate slot blots of pSP64- $\beta 1$ -Ref DNA and pSP64 DNA, under excess probe conditions, to assure the complete reaction of all β -tubulin RNA. The slots were exposed for autoradiography for 3 (left side), then 30 (right side) days. From densitometric measurements we calculated the β -tubulin RNA run-on per nucleus (left bar graph) relative to that of the 6-hr embryo, taken as unity. The run-on rates per embryo (right bar graph) relative to the 6-hr stage as unity were calculated from the number of cells per embryo (32, 200, and 500 at the 6-, 12-, and 23-hr stages, respectively).

Figure 9. Specificity of β -tubulin mRNA expression in pluteus ectoderm and endomesoderm tissue fractions. Pluteus larvae were fractionated into ectoderm (Ecto.) and endomesoderm (Endo.) tissue fractions. A mixture of total RNA (7 μ g/lane) from each of these fractions and 62.5 pg of 3 H-labeled reference RNA was electrophoresed, then blotted onto nitrocellulose. The gel blot was hybridized successively with the β 1 3'-specific probe and then the coding-region probe, as in Fig. 3. Exposures of 4 and 12 hr for the β 1 3'-specific probe and exposures of 4, 12, and 60 hr for the coding-region probe are shown.

spectively, over the endomesoderm. In contrast, there is little if any β 4 mRNA detectable in the ectoderm, resulting in a 13-fold enrichment of β 4 mRNA in the endomesoderm fraction. The tissue-specific enrichment of β -tubulin mRNAs is comparable to that of the Spec mRNAs (Bruskin et al. 1981), e.g., Spec 3 mRNA was recorded as being 14- to 20-fold enriched in the pluteus ectoderm.

The potential significance of β -tubulin tissue specificities Although the expression of certain individual β -tubulin genes is emphasized in specific tissues (Havercroft and Cleveland 1984), exclusive expression in a single cell type has not been demonstrated, except in the case of a divergent erythrocyte β -tubulin (Murphy and Wallis 1983). In the case of the sea urchin pluteus, widely different degrees of discrimination are apparent: Whereas the expression of β 2 and β 3 tubulin genes is higher by a small factor in the ectoderm compared with the endomesoderm fraction, the expression of the β 1 gene is almost exclusively ectodermal and that of the β 4 gene is, to the same degree, exclusively endomesodermal. The contrasting tissue specificities of the β 1 and β 4 tubulin genes may be considered in connection with the divergence in their respective coding region sequences, indicated by the melting differences of their hybrids with the β 1 coding probe. This probe includes the sequence (amino acids 288–377) encoding the amino-terminal portion of the carboxyl domain of the β -tubulin polypeptide (Mandelkow et al. 1985). Although this portion of the molecule does not include the extreme carboxyl terminus, which has been postulated to distinguish four isotypic classes of β -tubulin (Sullivan and Cleveland 1986), this region has some heterogeneity

among the amino acid sequences of the β -tubulin isotypes. The divergence in nucleotide sequences of β 1 and β 4 may, thus, indicate amino acid sequence differences and potentially distinct, tissue-specific β -tubulin isotypes. However, since a divergent chicken–yeast chimeric β -tubulin appears to function normally in microtubules of mouse 3T3 cells (Bond et al. 1986), various isotypes may not define specific microtubular functions. Unless isotype interchanges are shown to pose functional restraints in more highly differentiated cell types, tissue-specific expression of members of this gene family may be seen principally as a means for specific quantitative regulation.

Certain similarities in developmental and tissue-specific expression suggest that there may be some degree of coordination between β -tubulin and actin genes (Cox et al. 1986; Lee et al. 1986), both of which encode cytoskeletal proteins. The CyIIIa actin and β 1 tubulin mRNAs are similar in abundance and are highly enriched in the pluteus ectoderm. They have similar programs of developmental accumulation, with β 1 tubulin mRNA beginning to accumulate between the morula and early blastula stages and CyIIIa actin appearing some 2–3 hr later in the early blastula (Shott et al. 1984; Nemer 1986). The tissue specificity of CyIIa actin mRNA, which appears in the mesenchyme in the gastrula and in the gut of the pluteus (Cox et al. 1986), resembles that of β 4 tubulin mRNA, insofar as the latter is found in the pluteus endomesodermal tissue fraction comprising both structures. Moreover, both of these mRNAs, which are relatively rare among the transcripts of their respective families, do not accumulate until later embryonic stages: CyIIa actin mRNA after the mesenchyme blastula stage, and β 4 tubulin mRNA in the late-stage gastrula. The appearance of β 4 mRNA occurs long after cytoplasmic cables containing microtubules have arisen between mesenchyme cells and the formation of spicules (Gibbons et al. 1969). Since tubulin synthesis is conspicuously absent from these mesenchyme cells (Harkey and Whitely 1983), it is likely that β 4 tubulin mRNA is also absent and is present only in the gut of the late-stage embryo. Although precise localization of individual β -tubulin gene expression awaits the construction of additional gene-specific probes, our results support the hypothesis that certain members of these respective cytoskeletal gene families are coordinated in their tissue-specific expression.

Conclusions

A unique β -tubulin gene (β 1) has been identified among a total of 9–12 β -tubulin genes in the *S. purpuratus* genome, through the use of β 1-specific 3' untranslated and common β -tubulin coding-region probes derived from a previously sequenced β -tubulin cDNA, Sp- β 1 (Harlow and Nemer, in prep.).

At least four different β -tubulin RNAs have been found in *S. purpuratus* embryos. A 1.8-kb β 1-tubulin mRNA was detected by the β 1 3'-specific probe, while another 1.8-kb mRNA, designated β 2, and the 2.5-kb β 3

and 3.0-kb $\beta 4$ mRNAs were detected by the coding-region probe. Thermal denaturation studies utilizing the coding-region probe indicate that these RNAs are transcribed from at least three different β -tubulin genes.

The β -tubulin mRNAs were quantified by probing them on gel blots together with known molecular equivalents of a reference RNA. Although the $\beta 1$, $\beta 2$ and $\beta 3$ mRNAs display peak concentrations in the blastula and pluteus stages, only the concentration of $\beta 3$ is higher in the pluteus than in the blastula. In contrast, $\beta 4$ mRNA, the lowest in abundance, is not detected until the gastrula stage. The concentrations of total β -tubulin mRNA are directly correlated with the rates of β -tubulin protein synthesis in the early stages. The total of about 3×10^5 molecules of β -tubulin mRNA in the blastula would support a calculated rate of β -tubulin protein synthesis of about 3.6 pg/hr per embryo, which is substantially higher than the value of 0.4 pg/hr per embryo in the cleavage-stage embryo.

An increased rate of β -tubulin gene transcription during the cleavage–early blastula transition indicates that the early blastula-stage accumulation of β -tubulin mRNA is transcriptionally regulated. However, since the β -tubulin gene transcription rate per embryo does not change during subsequent development through the blastula stages, the observed decrease in the level of β -tubulin mRNA during this period cannot be attributed to a change in the rate of synthesis, but to post-transcriptional events. We conclude that there is a developmental shift in the predominant mode of regulation from transcriptional to post-transcriptional which probably involves a decrease in the stability of the β -tubulin mRNAs.

The β -tubulin mRNAs display different types and degrees of tissue specificity. The $\beta 2$ and $\beta 3$ mRNAs are moderately enriched (respectively, five- and threefold) per unit RNA mass in the pluteus ectoderm relative to the endomesoderm tissue fraction. However, $\beta 1$ mRNA, 17-fold higher in the ectoderm, is highly ectoderm specific. In contrast, $\beta 4$ mRNA is 13-fold enriched in the endomesoderm. Therefore, $\beta 1$ and $\beta 4$ mRNAs represent unusual degrees of exclusive expression of β -tubulin genes in different embryonic tissue types. Similarities in developmental profiles and tissue specificities between these β -tubulin mRNAs and counterpart transcripts of the actin gene family suggest that counterpart genes in these cytoskeletal protein families may be related in their modes of regulation in the *S. purpuratus* embryo.

Materials and methods

Embryos and embryo RNA

Conditions for the culture of *S. purpuratus* embryos in synthetic seawater have been described previously (Nemer et al. 1984). The procedure of McClay and Chambers (1978) was used for the preparation of ectodermal and endomesodermal tissue fractions from plutei. Total RNA was extracted from embryos or tissue fractions, as described by Nemer et al. (1984). RNA sizes were determined from their mobilities on Northern blots by comparison with sea urchin 18S and 26S rRNAs, 1.8 kb and 3.6 kb, respectively (Bieber et al. 1981), detected with Meth-

ylene Blue (Maniatis et al. 1982) and a synthetic reference RNA (931 bases, described below), detected by hybridization with labeled probe.

Bacteria and plasmids

The $\beta 1$ cDNA, which has been sequenced (Harlow and Nemer, in prep.) was obtained from the pSpB cDNA library in pBR322, representing *S. purpuratus* blastula polysomal RNA (Shepherd et al. 1983). This 1.3-kb cDNA, previously designated Blast J1 (Nemer 1986), was subcloned into the *Pst*I site of pUC19 by standard procedures (Maniatis et al. 1982) using competent JM83 cells (Morrison 1979). A coding-region probe was prepared by ligating the *Sac*I–*Sac*I fragment of this pUC19– $\beta 1$ plasmid into *Sac*I-digested pSP64 plasmid. The plasmid orientated to produce an anti-sense probe was designated pSP64– $\beta 1$ -coding. A $\beta 1$ -specific probe, representing its 3' untranslated sequence, was prepared through a series of subcloning steps, starting with the *Eco*RI–*Bam*HI fragment from pUC19– $\beta 1$. This fragment was inserted into *Eco*RI–*Bam*HI-digested pUC19. From this insert an *Eco*RI–*Pst*I fragment was obtained, digested with *Taq*I, subsequently ligated to *Eco*RI–*Acc*I-digested pUC19, and finally subcloned into pSP65, using the *Eco*RI–*Hind*III sites. This plasmid was designated pSP65– $\beta 1$ 3'. To prepare the template for synthesizing the reference RNA, the *Eco*RI–*Bgl*II fragment was isolated from pBR322– $\beta 1$ and ligated to *Eco*RI–*Bam*HI-digested pSP64. This plasmid was designated pSP64– $\beta 1$ -Ref. Plasmids were isolated by the alkali lysis method (Birnboim and Doly 1979) and large-scale preparations were further purified by equilibrium cesium chloride density gradient centrifugation.

Synthesis and isolation of RNA probes

*Hind*III-digested pSP65– $\beta 1$ 3' and *Eco*RI-digested pSP64– $\beta 1$ coding were used to synthesize run-off transcripts as anti-sense probes with SP6 RNA polymerase, as described by Melton et al. (1984), except that the final reactions included 10 mM NaCl, 11 μ M [α - 32 P]UTP (410 Ci/mmol, Amersham), 75 μ g/ml of linearized template DNA, and 375 U/ml of SP6 RNA polymerase (Promega Biotec). Reaction mixtures (20–40 μ l) were incubated at 40°C for 2 hr. Yeast tRNA to a concentration of 5 mg/ml and RNase-free DNase (Promega Biotec) to a concentration of 1.5 U/ μ g of template DNA were added and the reactions incubated at 37°C for 20 min. Following extraction with phenol-chloroform, the [32 P]RNA was ethanol precipitated. To separate the full-length transcripts from longer, plasmid-directed transcripts, which apparently hybridize to sea urchin rRNA, and any incomplete transcripts, the precipitated RNA was dissolved in 50% formamide, 5 mM EDTA, 0.05% xylene cyanol, 0.05% Bromophenol Blue, heated to 65°C for 5 min and electrophoresed on a 3% acrylamide–8 M urea gel. Brief autoradiography revealed the position of the full-length transcript. This portion of the gel was cut out and the RNA eluted into 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% SDS for 12–16 hr at 70°C with several changes of buffer. Typically, 250 ng of gel-purified probe with a specific activity of 7×10^8 to 9×10^8 cpm/ μ g were obtained.

Synthesis and isolation of the synthetic reference RNA

The reference RNA, a sense strand containing the sequences of the $\beta 1$ 3'-specific and coding-region probes, was synthesized as a run-off transcript from *Eco*RI-digested pSP64– $\beta 1$ -Ref DNA. The procedure was the same as above, except that [8 - 3 H]GTP (13 Ci/mmol, ICN) was diluted with unlabeled GTP to a spe-

cific activity of 5.2 Ci/mmol and used at a final concentration of 100 μ M. After phenol-chloroform extraction, the RNA was purified from unincorporated nucleotides by chromatography on Bio-Gel 1.5 M. The peak fractions in the excluded volume were ethanol precipitated. Typically, 3–4 μ g of [3 H]RNA with a specific activity of 7.5×10^6 disintegrations/ μ g was obtained. The RNA was dissolved in sterile water and stored at -70°C . Dilutions were made in 0.2 mg/ml yeast tRNA. For accurate quantification the [3 H]RNA of the stated specific activity was stable for greater than 2 weeks and less than 4 weeks; however, the stability can be substantially increased by employing a lower specific activity.

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 1 μ l of serial dilutions of [3 H] reference RNA was added to the sample mixture prior to heating to 65°C . Northern blots were prehybridized 3–5 hr at 44°C in the presence of 50% formamide, $5 \times$ SSC, 50 mM phosphate buffer (pH 7), $7.5 \times$ Denhardt's solution (Denhardt 1966), 0.1% sodium pyrophosphate, 0.1% SDS, 1 mM EDTA, 0.2 mg/ml yeast RNA, and 0.1 mg/ml denatured salmon sperm DNA. Hybridization with RNA probes was performed for 12–14 hr at 44°C in fresh prehybridization solution in which the Denhardt's solution was decreased to $5 \times$ and the yeast RNA was increased to 0.3 mg/ml. Typically, each gel blot (about 170 cm^2) was incubated with 10^7 cpm of probe. High-stringency washes were performed in $0.1 \times$ SSC, 0.1% sodium pyrophosphate, 0.1% SDS at 70°C . Routinely, gel blots were initially hybridized with the $\beta 1$ 3' probe, washed, and autoradiographed. After the probe was removed from the filter in 0.01% SDS at 95 – 100°C for 10 min, the gel blot was hybridized with the coding-region probe. The number of $\beta 1$ RNA molecules was calculated directly by comparing the $\beta 1$ 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. Similarly, using appropriate exposures, the number of $\beta 3$ and $\beta 4$ RNA molecules was calculated by comparing the coding-probe signals in the 2.5-kb band ($\beta 3$) and the 3.0-kb band ($\beta 4$) with the coding-probe signal in the reference RNA band. Since $\beta 2$ RNA was not resolved from $\beta 1$ RNA when probed with the coding probe, $\beta 2$ 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 $\beta 1$ RNA determined using the $\beta 1$ 3'-specific probe. To correct for uniformity of input RNA per lane, the coding-region probe was removed from the blots, which were then hybridized to nick-translated pSpBc12, equivalent in its restriction map to the mitochondrial ribosomal RNA clone SpP389 (Jacobs et al. 1983). The signal of the 1.4-kb mtRNA band assayed densitometrically served as a gauge of total RNA, since the content of mtRNA is essentially constant during development. The corrected 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).

Analysis of RNA levels by solution hybridization

Tracer excess hybridizations were performed with the purified [32 P]RNA probes described above and varying amounts of total RNA isolated from 20-hr blastulae. The procedure described by Lee et al. (1986) was followed, except that hybridization was in the presence of 50% formamide, $2.5 \times$ SSC, 50 mM phosphate buffer (pH 7), 1 mM EDTA at 44°C for 20 hr and unhybridized tracer was digested with 65 μ g of RNase A at 37°C for 1 hr.

Backgrounds of 0.26% and 0.19% of input for the $\beta 1$ 3' probe and the $\beta 1$ -coding probe, respectively, were subtracted. Slopes of the RNase-resistant cpm versus the total RNA input (microgram) were calculated by a linear least-squares analysis of the data points. To calculate the mass of mRNA (picograms/embryo), we used the equation, $xy/\alpha \gamma \delta$, where x is the slope of the titration curve (cpm of probe/ μ g of total RNA); y , the mass of total RNA/embryo (3.0 ng, Nemer et al. 1984); α , the ^{32}P scintillation counting efficiency (97.3%); γ , the specific activity of the probe (disintegrations/min per pg); and δ , the fraction of mature mRNA represented on the probe. The $\beta 1$ 3'-specific probe represents 183 bases of a 1.8-kb RNA, while the coding probe represents 277 bases of the average β -tubulin mRNA of 2.0 kb. The number of molecules/embryo is derived by dividing the picograms of RNA/embryo by the molecular weight of the RNA (picograms/molecule). The use of a higher salt concentration ($5 \times$ SSC) or higher temperature (50°C) did not alter the solution hybridization results with the $\beta 1$ 3' probe.

Genomic DNA blots

Separate sperm DNA preparations were made from several individuals according to the procedure described by Lee et al. (1984). Aliquots of these DNA samples were digested to completion with various restriction enzymes and electrophoresed on 0.7% agarose gels prior to blotting onto nitrocellulose filters by the method of Southern (1975). These filters were prehybridized as described above for the Northern blots. Hybridization with the [32 P] RNA probes was as described above, except that it was extended to 24 hr. Initial stringency washes were in $0.5 \times$ SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 60°C . For the $\beta 1$ 3'-specific probe high-stringency washes were in $0.3 \times$ SSC at 65°C . For the coding probe, moderate stringency washes were in $0.3 \times$ SSC at 65°C , while high-stringency washes were in $0.1 \times$ SSC at 70°C .

Determination of the number of β -tubulin genes

*Hind*III-digested SP64- $\beta 1$ -Ref DNA was diluted using *Hind*III-digested λ DNA as carrier so that amounts of plasmid DNA equivalent to 1, 2, 3, 5, 10, 15, and 20 copies of β -tubulin sequence contained in 1 μ g of *S. purpuratus* genomic DNA would be loaded per slot as standards. Assuming 0.89 pg of DNA as the haploid genome of *S. purpuratus* (Hinegardner 1974), one haploid genome equivalent is equal to 4.86 pg of pSP64- $\beta 1$ -Ref. Genomic DNA from two of the individuals described above was digested with *Pst*I. Replica filters were prepared, except that the filter for the coding probe contained 0.5 and 1 μ g of each sample genomic DNA, while the filter for the $\beta 1$ 3'-specific probe contained 1 and 3 μ g of each sample genomic DNA. All DNA samples were denatured in 0.3 M NaOH at 95°C for 1 hr, chilled on ice, neutralized with an equal volume of 2 M ammonium acetate, and immediately loaded onto nitrocellulose slot blots. The filters were baked for 2 hr at 80°C in vacuo, prehybridized as described above for the Northern blots, hybridized to ^{32}P -labeled RNA probes for 24 hr at 44°C , washed at the indicated stringencies for the genomic blots, and autoradiographed. The genomic samples were compared with the plasmid standards by scanning densitometry.

Nuclear run-on transcription

Nuclei were isolated from 6-, 12- and 23-hr embryos as described by Marzluff and Huang (1984). The purified nuclear suspension, containing 3.2×10^7 nuclei from each stage, 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]. The rate of incorporation of [α - 32 P] UTP in RNA was monitored through binding to DE-81 filters (Whatman) in 0.5 M NaHPO₄, and the labeled RNA was purified as described (Wilkinson and Nemer 1987). To compare the relative rates of transcription of β -tubulin genes in nuclei from 6-, 12-, and 23-hr embryos, equal amounts of radioactivity of the recovered nuclear RNA were separately hybridized to an excess amount (5 μ g) of linearized SP64- β 1-Ref plasmid, immobilized on nitrocellulose as a slot blot (see above). This hybridization was in 30% formamide, 5 $\times$ SSC, 50 mM sodium phosphate (pH 7.0), 5 $\times$ Denhardt's solution, 0.1% SDS, 0.1% sodium pyrophosphate, 0.2 μ g/ml poly(A), 0.5 mg/ml yeast RNA, 0.1 mg/ml denatured salmon sperm DNA, at a total volume of 0.5 ml at 44°C for 72 hr. Following this, the filters were washed at a final stringency of 0.3 $\times$ SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 50°C, then treated with 20 μ g/ml of RNase A in 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA at 37°C for 30 min, rewashed in 0.3 $\times$ SSC at 50°C, and subjected to autoradiography. RNase treatment served to reduce noise and to increase the effective stringency, as gauged by treatment of Northern blots with RNase under similar conditions, to a level sufficient to reduce nonspecific hybridization of RNA with SP6 vector sequences, but allow detection of all four β -tubulin RNAs by the coding sequences contained in the SP64- β 1-Ref probe.

Acknowledgments

We are grateful to Elizabeth Watkins and Margaret Campbell for their excellent technical assistance. This investigation was supported by grants from the U.S. Public Health Service, [HD-04367 (M.N.), CA-06927, and RR-05539 (I.C.R.)] and by an appropriation from the Commonwealth of Pennsylvania. P.H. is a postdoctoral trainee supported by National Institutes of Health grant CA-09035-11.

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Genes Dev. 1987, **1**:

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