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Multiple polymorphic α- and β-tubulin mRNAs are present in sea urchin eggs

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ABSTRACT **Multiple α- and β-tubulin RNAs were found in the mature unfertilized eggs of the sea urchin *Lytechinus pictus*. The α-tubulin RNAs were polymorphic in number, size, and relative amounts in the eggs of different females. Five to seven different size classes [1.75–4.2 kilobases (kb)] were detected on RNA gel blots. All egg preparations contained variable amounts of 1.8- and 2.25-kb β-tubulin RNAs, and a few of them contained an additional 2.9-kb β-tubulin RNA. The total amount of α-tubulin RNA did not always parallel that of β-tubulin RNA. A portion of all of the various α- and β-tubulin RNAs were polyadenylylated. RNase H digestions ruled out the possibility that some of these RNAs represented a single transcript bearing different lengths of 3′ poly(A). One class of α-tubulin RNAs (2.4–2.65 kb) was reduced to 2 kb by RNase H, suggesting the presence of internal oligo(A) regions. All of the egg β-tubulin RNAs sedimented as free ribonucleoprotein particles. Only a small portion of the 1.75- to 3.6-kb α-tubulin RNAs, but most of the 4.2-kb α-tubulin RNA, were found on polysomes before fertilization. In the 30-min embryo, small amounts of each of the various α- and β-tubulin RNAs were recruited onto polysomes. Thus, each of the multiple polymorphic α- and β-tubulin RNAs in the egg represent translationally competent mRNA.**

The sea urchin egg contains large stockpiles of structural proteins and enzymes that help sustain the rapid cleavage divisions of the early embryo (1–6). Little is known about the kinetics of synthesis of these proteins during oogenesis. In the first readily available stage, the mature unfertilized egg, the overall rate of protein synthesis is low (reviewed in refs. 7–9).

α- and β-tubulins synthesized during oogenesis (10) make up 1%–5% of the egg protein (11, 12). This pool provides most of the tubulins for the first mitotic spindle (13) and the cilia of the blastula (14). Despite this excess, tubulin synthesis increases after fertilization, roughly in parallel with overall protein synthesis (6, 15). Questions about how many different α- and β-tubulins are made by the sea urchin and whether any of these are specific for mitotic spindles, cilia, flagella, or cytoskeleton remain unanswered. Several studies indicate the existence of multiple organelle-specific tubulins (16), including four α- and four β-tubulin variants (17, 18) in the egg, but they do not distinguish between primary sequence variations and post-translational modifications. The sea urchin genome contains 9–13 α- and β-tubulin sequences (19). At least two different α- and three different β-tubulin genes are expressed at specific developmental times (see ref 20; unpublished data), but the types of tubulins encoded by these genes, and possibly by others, are unknown.

In addition to stored proteins, the sea urchin egg contains a large pool of untranslated mRNA (21) and hnRNA-like sequences (22). Within 5 min of fertilization, there is a 10- to 20-fold increase in the rate of protein synthesis, which is due exclusively to the recruitment of stored mRNA. The functions of most translationally activated mRNAs are unknown. The tubulins are among the few proteins that have been identified as products of these stored, maternal RNA sequences (23) [others are histones (24), actins (25), cyclins (26), and ribonucleotide reductase (ref. 27; unpublished data)]. In this context, characterization of the egg tubulin transcripts, their translational fates, and the types of tubulins they encode are of interest. Here, we have identified several size classes of translationally competent maternal tubulin mRNAs, some of which were distinguished by their long sizes and by polymorphism in size and abundance.

MATERIALS AND METHODS

Preparation of Egg RNA. *Lytechinus pictus* (Pacific Biomarine, Venice, CA) eggs collected and treated as described (19) were lysed in 0.35 M NaCl/1 mM EDTA/10 mM Tris·HCl, pH 8/2% $NaDodSO_4$/7 M urea. Total nucleic acids were purified by phenol/chloroform/isoamyl alcohol (25:24:1) extractions. Isolation of testis RNA, and fractionation of RNA on oligo(dT)-cellulose into poly(A)$^+$ and poly(A)$^-$ RNAs have been described (19).

Treatment of RNA with RNase H. RNA samples were treated with RNase H as described by the supplier (Bethesda Research Laboratories).

Polysome Gradients. Eggs and embryos were homogenized in 0.3 M glycine/250 mM KCl/3 mM magnesium chloride/40 mM Hepes, pH 7.3, and supernatants were centrifuged at 12,000 × *g* on 11 ml of 15%–40% (wt/vol) sucrose gradients (made in 0.5 M KCl/6 mM magnesium chloride/1 mM EDTA/10 mM Hepes, pH 7.4) for 95 min at 40,000 rpm in a SW41 rotor (28, 29). In some experiments, the RNA was released from polysomes prior to centrifugation by treatment with 2 mM puromycin dihydrochloride or 30 mM EDTA (30, 31).

RNA Gel Blot Analysis. RNA gel blotting and hybridization were carried out as described (29). Sea urchin and *Escherichia coli* rRNA (32) and endonuclease restriction fragments of λ and M13 DNAs were used as molecular weight markers.

RESULTS

Polymorphism of α- and β-Tubulin RNAs in the Sea Urchin Egg. The α- and β-tubulin RNAs present in total RNA isolated from mature eggs of 20 individual female sea urchins were analyzed by RNA gel blot hybridization. Both cDNA and genomic DNA probes were used. pα2 is a testis cDNA clone that contains 483 bases of COOH-terminal coding and 300 bases of 3′ untranslated sequence (20). The non-coding portion cross-hybridizes only with testis RNA (51). G-pα21B2 is a subcloned 1.5-kb *Bgl* II fragment of the genomic α-tubulin clone λLpT21 (19), which contains α-tubulin coding sequence and a 3′ non-coding sequence that differ from those in pα2; it does not contain any non-tubulin-specific se-

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Abbreviation: kb, kilobase(s).

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quences (51). pβ2 is a testis cDNA clone that contains 392 bases of COOH-terminal coding and 393 bases of 3′ untranslated sequence (20). G-pβ6E2 is the subcloned 2.8-kb *Eco*RI fragment of the genomic β-tubulin clone λLpT6 (19) that contains, in addition to coding sequences, non-coding sequences different from those in pβ2 (51).

The α-tubulin RNA sequences in the egg gave very complex hybridization patterns that varied among individual females. The 10 most dissimilar patterns are shown in Fig. 1 (*Left*). pα2 hybridized to several RNA size classes ranging from 1.75 to 3.6 kb. The 1.75-kb RNA was present in small amounts in all preparations. While all samples contained 3–5 larger RNAs (≥2.2 kb), the presence, relative abundance, and exact sizes of these RNAs varied considerably among different females. G-pα21B2 hybridized to the same RNA bands, but gave higher overall intensities of hybridization. Also, the larger RNA species showed relatively higher hybridization intensities with the genomic probe than with the cDNA probe. This difference is probably due to hybridization between non-coding sequences contained in the larger RNAs and G-pα21B2, but absent from the smaller RNAs and pα2.

In contrast to the α-tubulin RNA patterns, the same RNA samples exhibited limited variations in their β-tubulin RNAs (Fig. 1, *Right*). For example, pβ2 usually hybridized to only two RNAs, 1.8 and 2.25 kb. In all samples, the 2.25-kb β-tubulin RNA was more abundant than the 1.8-kb RNA but the ratio of the two RNAs varied in different females. In one case, a third (2.9-kb) RNA was prominent. The patterns seen with G-pβ6E2 were essentially identical to those obtained with pβ2. Just as for α-tubulin, the β-tubulin genomic probe gave higher overall intensities of hybridization than the cDNA probe. Indeed, with the genomic probe a very faint 2.9-kb β-tubulin RNA was detectable in samples 3 and 5 in some RNA blots (as seen in Fig. 3).

In addition to these qualitative differences, quantitative differences were noted (Fig. 1). Some egg batches contained as much as 5 times more α- or β-tubulin RNAs than others (data not shown). In some cases, the amounts of α- and β-tubulin RNAs varied in parallel, while in others they did not.

Except for the 1.75-kb α-tubulin RNA and 1.8-kb β-tubulin RNA, the tubulin transcripts in the egg are considerably longer than the 1.6–2 kb expected for mature tubulin mRNAs (20, 33–36). The large RNAs seen here could represent various processing intermediates of one (or more) primary transcripts, they could be mRNAs transcribed from different alleles or different genes, or they could represent a single sequence bearing different length 3′ poly(A)s. In the next sections, we describe the results of experiments designed to test some of these possibilities.

Polyadenylylation of Maternal α- and β-Tubulin mRNAs. Poly(A)-containing RNA was isolated from several egg RNA preparations and was analyzed by gel blot hybridization. A portion of all size classes of both α- and β-tubulin RNAs are polyadenylylated (Fig. 2). Not all tubulin RNAs were equally represented in the poly(A)$^+$ fraction. For example, the 2.65-kb α-tubulin RNA and the 2.25-kb β-tubulin RNA were more abundant in poly(A)$^+$ RNA. The largest α-tubulin RNAs (3.8–4.2 kb) were scarcely detectable in total or in poly(A)$^-$ RNA, either because they are mostly poly(A)$^+$ or because of interference by the large amounts of comigrating rRNA.

The possibility that different size transcripts could represent a single RNA carrying a series of different length poly(A) tracts was tested by comparing the sizes of these RNAs after *in vitro* removal of poly(A) using RNase H (37, 38). With one exception, the α- and β-tubulin RNAs did not show a significant shortening after RNase H treatment, suggesting that each of these RNAs contained short 3′ poly(A)s (Fig. 3). Thus, differential adenylylation cannot account for either the multiplicity or polymorphism of egg tubulin RNAs.

For one α-tubulin class, whose size varied from 2.4 to 2.65 kb among different samples, a dramatic size reduction of 2 kb did occur after oligo(dT)-dependent RNase H treatment. This is most obvious in samples 4, 5, 3, and 10 (Fig. 3), in which these RNAs are relatively more abundant. These particular transcripts contain either unusually long 3′ poly(A) tracts or, more likely, internal oligo(A) sequences. Such internal oligo(A) runs have been described for sea urchin egg, *Xenopus* oocyte, and several nuclear and viral RNAs (39–41). However, we have not been able to find 0.4- to 0.65-kb (or smaller) fragments by hybridization with the whole α genomic probe λLpT21, which contains ≈10.5 kb of sea urchin DNA. Of course, it is possible that this probe does not contain these particular gene sequences.

Translational Competence of the Egg's Various Tubulin RNAs. If any of the different sized tubulin RNAs in the egg were mature mRNAs, we would expect them to be recruited onto polysomes without any significant size change. If some of these RNAs were incomplete processing intermediates, we would expect them to be reduced to the size of mature mRNA before entering polysomes. To investigate whether any of these sequences were associated with polysomes, 12,000 × *g* supernatants of egg or 30-min embryos were separated into polysomal and non-polysomal fractions by cen-

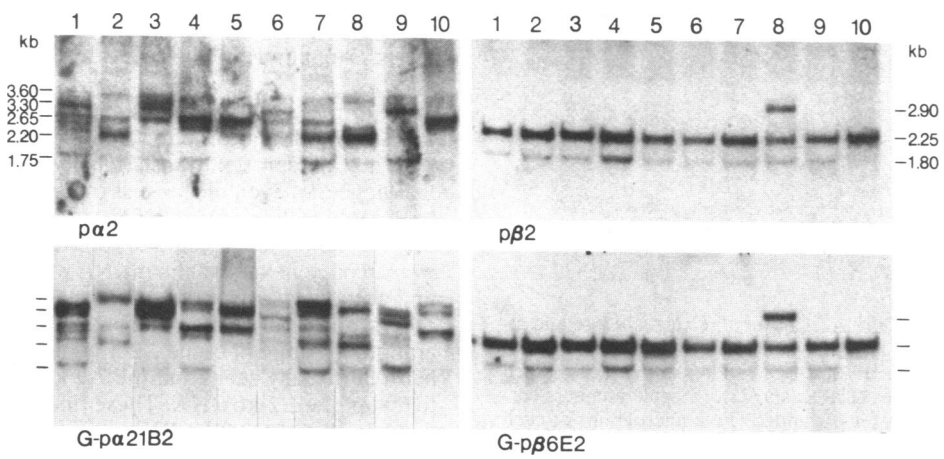

FIG. 1. Autoradiograms of gel blotted α- and β-tubulin transcripts in 10 different preparations of sea urchin egg RNA. Five-microgram aliquots of total RNA isolated from mature eggs of 10 different female sea urchins were electrophoresed through a 1.5% agarose gel, transferred onto nitrocellulose filters, and hybridized with ^{32}P-labeled tubulin cDNA probes (pα2, pβ2) and genomic DNA probes (G-pα21B2, G-pβ6E2).

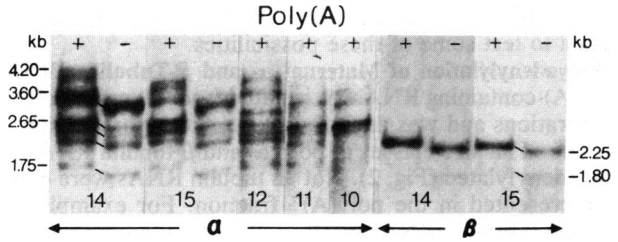

FIG. 2. Poly(A)$^+$ and poly(A)$^-$ tubulin RNAs in unfertilized eggs. Poly(A)$^+$ (0.5 μg) and poly(A)$^-$ RNA (5 μg) samples isolated from pooled eggs of one (lane 10), two (lanes 14 and 15), or three (lanes 11 and 12) animals were gel blotted and hybridized with probes G-pα21B2 (*Left*) and G-pβ6E2 (*Right*). For samples 10, 11, and 12 only poly(A)$^+$ fractions are shown. Poly(A)$^+$ and poly(A)$^-$ RNA of similar sizes migrated differently in the gels (as indicated by connecting lines), because of their widely different content in rRNA.

trifugation through sucrose gradients. The RNA present in each of the seven gradient fractions was then examined by RNA blot analysis (Fig. 4). In the egg, most of the (1.75–3.6 kb) α-tubulin RNA was found in fractions 6 and 7—that is, in the region of free ribonucleoprotein particles (RNPs). A small portion of the RNA was detected in fractions 3, 4, and 5, which contained large RNPs and small polysomes. The longest (4.2 kb) α-tubulin RNA, which was barely detectable in overloaded total RNA samples (lanes T), sedimented mainly in the polysome region in eggs. A very small fraction of all α-tubulin RNAs, and more of the largest α-tubulin RNA, pelleted during centrifugation.

Which of these RNAs that were found in the polysome region in eggs represent mRNAs engaged in translation and which represent heavy RNPs that merely co-sedimented with polysomes? We used EDTA- and puromycin-mediated polysome disruption to distinguish between these alternatives. The former method is often used to test for functional mRNA–polysome associations, but it suffers from the drawback that EDTA can also strip off some proteins from RNPs, thus decreasing their sedimentation rate (31). Puromycin, an aminoacyl-tRNA analogue, releases mRNAs from polysomes by a more specific method. Fig. 4 shows that all of the α-tubulin RNAs found in the polysome region sedimented more slowly (in the RNP region) after EDTA or puromycin treatment, indicating that these RNAs were indeed being translated on polysomes. Puromycin failed to release any significant amount of RNA from the pellet, suggesting that most of this RNA was associated in some way with other heavy, non-polysomal cellular structures, such as cytoskeleton, or was trapped nonspecifically by such structures.

Within 30 min of fertilization, more of each of the α-tubulin RNAs was recruited onto polysomes. The longest (4.2 kb) α-tubulin RNA sedimented on polysomes that were significantly heavier than those found in the egg. EDTA and puromycine treatments released these RNAs from polysomal fractions, showing that all of these RNAs were indeed associated with polysomes. Both reagents also released most of the pelleted RNAs, indicating that they were bound on very heavy polysomes at this stage. Thus, all of the various α-tubulin RNAs in the egg, even the longest, were capable of being actively translated and are thus bona fide mRNAs.

Unlike α-tubulin RNAs, none of the β-tubulin RNAs were detected on polysomes before fertilization (Fig. 4). In the 30-min zygote, a small portion of the 2.25-kb β-tubulin RNA was found on polysomes. The minor 1.8-kb β-tubulin RNA was not detected in polysomes in these early embryos. Other studies showed that this RNA could be seen on polysomes at later cleavage stages when most α- and β-tubulin RNAs are translated (unpublished data). Thus, both the 1.8- and 2.25-kb β-tubulin RNAs represented translatable mRNAs.

Because we encountered so much polymorphism in the α-tubulin mRNAs in different egg preparations, we repeated these polysome gradient analyses with four more egg and embryo preparations taken from four different females. In all cases, the results for both α- and β-tubulin RNAs matched those shown in Fig. 4 (data not shown).

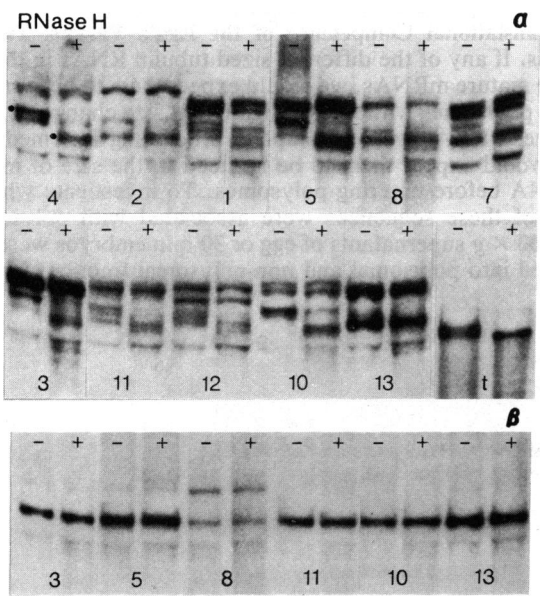

FIG. 3. Comparison of the sizes of tubulin RNAs before and after treatment with RNase H. Total RNA was isolated from eggs of individual females (samples 1, 2, 3, 4, 5, 7, 8, and 10) or eggs pooled from three different females (11, 12, and 13). (Sample numbers correspond to those shown in Figs. 1 and 2.) Five micrograms of each RNA preparation was treated with RNase H. Untreated (−) and treated (+) samples were gel blotted and hybridized with G-pα21B2 or G-pβ6E2 probes. A testis cytoplasmic poly(A)$^+$ RNA (lanes t) was included as a positive control. Dots indicate the 2.65-kb RNA before and after RNase H treatment.

DISCUSSION

The findings reported here directly and unambiguously confirm the early conclusions of Raff *et al.* (23) that there is a store of tubulin RNA in the unfertilized sea urchin egg and that some of this RNA is recruited for translation soon after fertilization (15). Surprisingly, several size classes of α- and β-tubulin RNAs were detected and these varied among eggs obtained from different females. Five to seven different α-tubulin RNA size classes (1.75–4.2 kb), and two or three different β-tubulin RNA size classes (1.8, 2.2, and 2.9 kb) were seen.

All of the α-tubulin RNAs and two of the three β-tubulin RNAs were found on polysomes in the embryo, indicating that they all represent translationally competent mRNAs and that none (not even the longest α-tubulin RNA) belong to the hnRNA-like class of egg RNA described by other investigators (22, 32). The largest, 2.9-kb β-tubulin RNA might, however, represent a β-tubulin mRNA precursor. This RNA was undetectable in eggs from some females, barely detectable in others, and prominent in one batch of eggs. In this last preparation, the amount of the 2.2-kb β-tubulin mRNA was about half that usually seen, and the 2.9-kb band was about equal to that of the 2.2-kb RNA. These findings are consistent with the idea that the 2.9-kb RNA represents a precursor of the 2.2-kb RNA that is usually processed before egg maturation. However, we cannot exclude the possibility of polymorphic variation in transcription or accumulation from allelic or different genes, since none of the five egg preparations that we

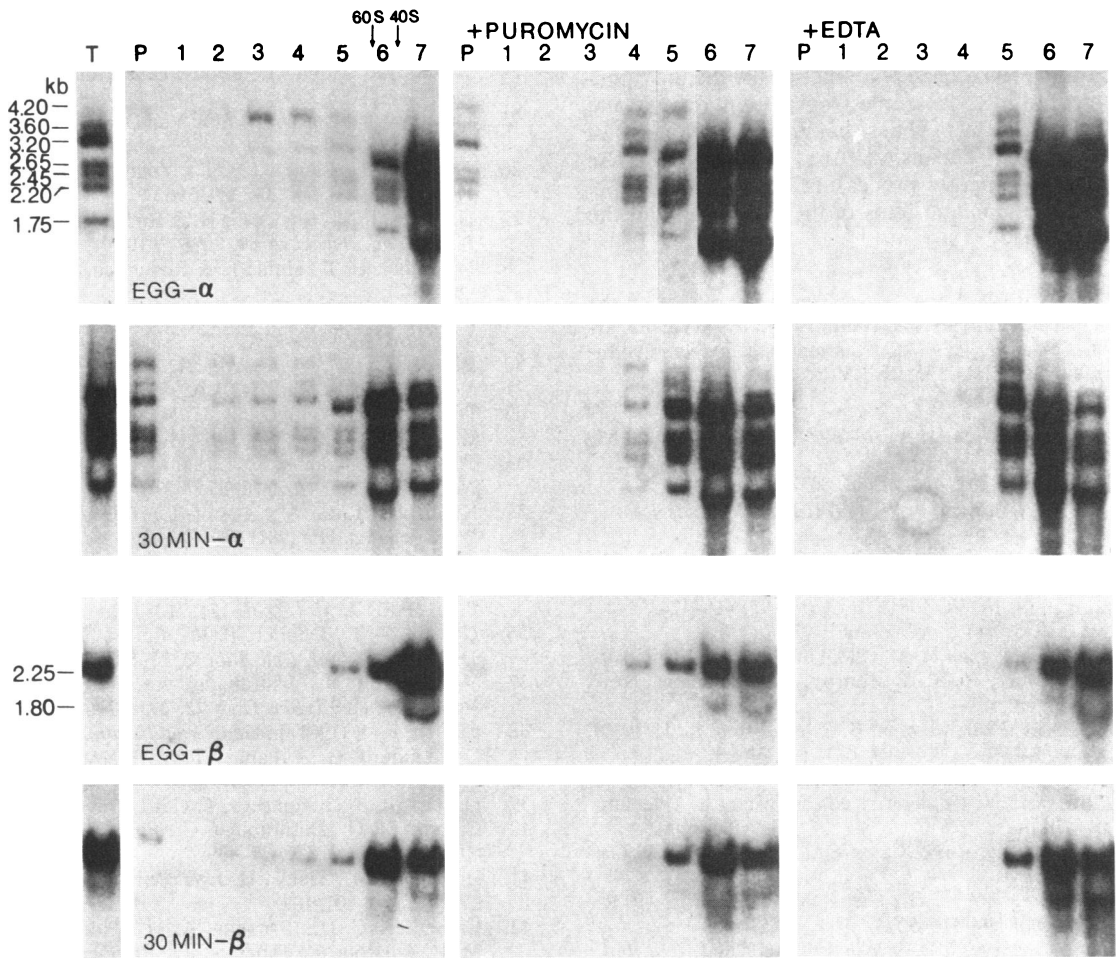

FIG. 4. Association of α- and β-tubulin RNAs with polysomes before and after fertilization. Cytoplasmic supernatants (12,000 g) of eggs and 30-min zygotes obtained from the same female were sedimented on sucrose gradients and fractionated onto seven fractions (lanes 1–7). RNA was extracted and an equal portion from each fraction was gel blotted and hybridized with G-pα21B2 or G-pβ6E2 probes (*Left*). Aliquots of the 12,000-g cytoplasmic samples (lanes T) and the gradient pellets (lanes P) were analyzed in parallel. The same samples were treated with puromycin (*Middle*) or EDTA (*Right*) prior to centrifugation. In all gradients, fraction 1 contained a smaller portion of material than the others. Different gradients contained similar, but not identical, amounts of 12,000-g cytoplasmic samples. Gel lanes 6 and 7 contained very high amounts of RNA, which resulted in the anomalous migration of some tubulin RNAs.

analyzed for translational activity contained detectable amounts of the 2.9-kb β-tubulin RNA.

Why are some of these RNAs so much longer than the length (1.6–2 kb) that would typically be needed to encode a 50,000- to 53,000-dalton protein? The results of the RNase H experiment rule out the possibility of long poly(A) tracts. Given that tubulin-coding sequence lengths are very conserved (20, 35, 36) and that all of the different urchin tubulin RNAs were recruited onto similar sized polysomes in the early embryo (Fig. 4), it is most likely that the extra lengths are part of long untranslated sequences. While such long non-coding sequences are not common, examples do exist for the sea urchin (42) and other animals (43). Indeed, long translationally active β-tubulin RNAs were recently reported for chicken (1.8–4 kb) (44), and human (1.8 and 2.6 kb) (45).

Another unresolved issue is whether the multiple tubulin mRNAs derive from a single gene or from multiple genes. Since there are 9–13 α- and β-tubulin sequences in the sea urchin genome (19), transcription of more than one gene is a possibility. However, many of these genes might be pseudogenes, similar to those described for humans and rats (45, 46). Alternative use of polyadenylylation sites and differential splicing pathways could also contribute to RNA heterogeneity. In fact, different sized β-tubulin mRNAs produced from alternative polyadeylylation sites have been found in human RNA (45), and different sized β-tubulin mRNAs transcribed from the same gene (or allele) have been identified in chicken embryo RNA (44) and in sea urchin testis RNA (20).

We were surprised by the extent of polymorphism in size, number, and relative abundance of α-tubulin mRNA. While there is a high degree (≈4%) of polymorphism in single copy DNA of individual sea urchins, this is more than 10 times the level estimated for protein sequences (47, 48). Polymorphism has also been seen for single copy DNA fragments that are complementary to sea urchin egg poly(A)$^+$ RNA (32) and in DNA restriction enzyme fragments that contain actin genes (49, 50). The tubulin RNAs provide a clear example of polymorphism in mature translationally active mRNAs of easily distinguished sizes. Considering the high conservation of tubulins (20, 35, 36), it is likely that this size polymorphism lies in the untranslated regions of the mRNAs.

We were also surprised by two instances of non-coordinate regulation of α- and β-tubulin mRNAs. First, the relative amounts of α- and β-tubulin mRNAs were not constant among different egg preparations. Second, a small portion of all α-tubulin mRNAs were found on polysomes before fertilization, whereas β-tubulin mRNAs were not. These results suggest that coordinate production of α- and β-tubulin proteins in microtubules occurs after translation of these particular mRNAs.

Thus, there is a complex pattern of maternal tubulin mRNAs in the unfertilized egg. This raises several questions. Does each mRNA encode a functionally distinct tubulin? If not, do the mRNAs come from multiple genes and have specific roles or from genes that are a consequence of the tubulin gene family expansion? Are the multiple mRNAs the products of alternative processing pathways? Finally, what are the developmental fates of these mRNAs and the proteins they encode?

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