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Expression of the U1 RNA gene repeat during early sea urchin development: Evidence for a switch in U1 RNA genes during development

(RNA processing/small nuclear RNA)

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ABSTRACT **The majority of the genes for U1 RNA are organized in tandemly repeated units in the sea urchin. To assess the level of expression of these genes in the sea urchin *Lytechinus variegatus*, we measured the transcription of sequences 3′ to the gene. The tandemly repeated U1 genes are expressed in morula and continue to be expressed at high rates until 2 hr after hatching, at which time the rate of expression of all the U1 genes and the tandemly repeated U1 genes declines sharply. By the gastrula stage the synthesis of total U1 RNA has declined by a factor of 8. The major tandemly repeated genes are inactive by this time, although other U1 genes remain active. The sequence of U1 RNA synthesized late in embryonic development differs from the sequence of U1 RNA encoded by the tandemly repeated set of U1 RNA genes, indicating that there must be other U1 RNA genes that are active late in embryonic development.**

The small nuclear RNAs (snRNAs) are essential for the biosynthesis of functional mRNAs (reviewed in ref. 1). Functional small nuclear ribonucleoproteins are required for expression of newly synthesized mRNAs during embryonic development. In sea urchin eggs, there are large amounts of U1 RNA (2, 3). This RNA remains in the cytoplasm during early embryonic development and may never function in mRNA synthesis in the embryo (3). Synthesis of U1 RNA is initiated in the morula stage (4) and the newly synthesized U1 RNA is found in the nucleus (3). The synthesis of U1 RNA may precede the synthesis of functional mRNA (3). The rate of synthesis of U1 RNA is high during morula and early blastula and then declines rapidly between blastula and gastrula (4).

The majority of the U1 RNA genes in sea urchins are organized in tandemly repeated units (2, 5, 6). There are two types of repeat units in the sea urchin *Lytechinus variegatus*, which are interspersed in a single large tandemly repeated unit (6, 7). Both repeat units encode the same U1 RNA, which is identical in sequence to the U1 RNA found in sea urchin eggs and early embryos (7). Transcription of the U1 gene initiates at the first nucleotide of the RNA and extends 3′ to the gene for at least 200 nucleotides *in vitro* (8, 9). The expression of a particular U1 gene can be measured by monitoring the transcripts that extend into the 3′ flanking regions (7).

Using gene-specific probes from the 3′ flanking region, we show here that the tandemly repeated genes are active early in embryogenesis with peak activity at 2 hr after hatching. The activity of the repeated genes then declines rapidly and the genes are silent by gastrula, even though U1 RNA is still being synthesized by the embryo. Two U1 RNAs are present in eggs, the major species encoded by the tandemly repeated gene set and a minor species presumably encoded by a second, less repeated, gene set. During development, the proportion of the minor U1 snRNA increases until it becomes the major U1 snRNA in the pluteus embryo. This suggests that there is another set of U1 RNA genes, which may be constitutively expressed, and which is the only U1 RNA synthesized in later embryonic stages. This is reminiscent of the situation for the sea urchin histone genes. The tandemly repeated α-histone genes are active early in embryogenesis and then are inactivated after blastula (10, 11), while the late histone genes are expressed throughout embryonic development and become dominant in later stages (12, 13).

MATERIALS AND METHODS

Growth of Embryos. *L. variegatus* were collected at the Florida State University marine laboratory and embryos were grown at 23°C as described (4). In all experiments, >95% of the embryos developed normally. For preparation of nuclei from embryos prior to hatching, the embryos were fertilized and grown in the presence of 1 mM *p*-aminobenzoic acid, which prevented hardening of the fertilization membrane.

Isolation of Nuclei and RNA Synthesis. Nuclei were prepared as described by Morris and Marzluff (14) from embryos at different developmental stages. For the preparation of nuclei up to 6 hr after hatching, a 12-liter culture of embryos was prepared and aliquots were removed for preparation of nuclei. The gastrula embryos were obtained from a separate culture grown from sea urchins obtained from the same population at the same time. Nuclei were stored in liquid nitrogen at a concentration of 10^9 nuclei per ml (8).

For RNA synthesis, nuclei were incubated for 30 min at 25°C as described (8). The standard conditions used were 500 μM each ATP, GTP, CTP and 50 μM [^{32}P]UTP (10 Ci/mmol; 1 Ci = 37 GBq), 90 mM KCl, 5 mM $MgCl_2$, 1 mM spermidine, 10 μM *S*-adenosylmethionine, 12.5% (vol/vol) glycerol, 0.5 mM EGTA, 1 mM dithiothreitol, and 25 mM Tris·HCl (pH 8). RNA synthesis was monitored by measuring the incorporation of radioactivity into trichloroacetic acid-precipitable material. ^{3}H nuclear RNA was obtained from mesenchyme blastula embryos grown continuously in the presence of [^{3}H]uridine (10 μCi/ml) from morula to harvesting.

RNA was extracted by the procedure of Penman and coworkers (15) except that after the incubation the nuclei were treated with DNase I (20 μg/ml for 1 min) at 25°C prior to extraction with phenol in the presence of 0.5% sodium dodecyl sulfate (pH 5.0 at 55°C). Free nucleotides were removed by Sephadex G-50 spin columns.

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Abbreviation: snRNA, small nuclear RNA.

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DNA Probes. *L. variegatus* 5S DNA, pLv305-U13, is a 1.0-kilobase fragment containing one copy of a 5S gene cloned into pUC13 (16). This fragment was subcloned into the single-strand-producing vector pUC118. Unique regions of pLvU1.1 and pLvU1.2 (7) were subcloned into M13mp8 and M13mp9. There was no sequence similarity between the two gene-specific probes.

Hybridization. RNA was hybridized to "dots" of the various single-stranded DNA probes immobilized on nitrocellulose essentially as described by Marzluff and Huang (17). Usually each of the 5S and unique DNA probes was hybridized with 1×10^6 cpm and the coding probe was hybridized with $2–3 \times 10^5$ cpm of [^{32}P]RNA synthesized *in vitro*. The hybridizations also contained 5×10^4 cpm of ^{3}H nuclear RNA as a control for hybridization efficiency. The amount of RNA bound to each dot was quantified by densitometer scanning of the autoradiograph and by liquid scintillation counting of each dot. The results were expressed as the fraction of input RNA that hybridized to the filter.

Primer-Extension Analysis. To determine the sequence of the different U1 variants, a primer was synthesized on an oligonucleotide synthesizer (Applied Biosystems, model 381B). The primer was end-labeled with $^{32}PO_4$ using polynucleotide kinase and annealed with nuclear RNA at 65°C for 1 hr in 0.25 M KCl/10 mM Tris·HCl/1 mM EDTA, pH 8.0, and then cooled to 32°C over a period of 4 hr. Avian myeloblastosis virus reverse transcriptase (5 units; Seikagaku Kogya, Tokyo), three deoxynucleotides (500 μM), and one dideoxynucleotide (500 μM) were added and the reaction mixture was incubated for 1 hr at 37°C. The nucleic acid was purified by extraction with phenol, recovered by centrifugation after precipitation with ethanol, and analyzed by electrophoresis on a 20% polyacrylamide/7 M urea gel. The gel was exposed to x-ray film and the amount of each RNA species was quantified by densitometry.

RESULTS

We have previously described the isolation and structure of two members of a tandemly repeated unit encoding U1 RNA genes from *L. variegatus* sperm DNA (2, 7). Each of these repeat units, pLvU1.1 and pLvU1.2, contains a single U1 gene. One of the types of repeat unit, pLvU1.2, is present in 4 times the copy number of the other (2). The sequence of these two genes is highly divergent, starting 30 nucleotides 3′ of the U1 coding region. Since we have previously shown that transcription extends 3′ to the end of U1 RNA in both sea urchin (7, 9) and mouse (18) nuclei, subclones were constructed from the unique regions 3′ to the cloned U1 repeat units (Figs. 1 and 2*A*). The use of the 3′ flanking sequence probes allows one to assess the activity of different members of a gene family encoding similar or identical RNAs and overcomes the difficulty of distinguishing small amounts of very similar transcripts. A similar approach using the differences in the 3′ untranslated region has been used to demonstrate that each of the genes of the actin gene family is regulated independently during sea urchin development (19). The activity of individual members of the mouse histone gene family has also been measured by assaying transcription of the flanking regions in isolated nuclei (20, 21).

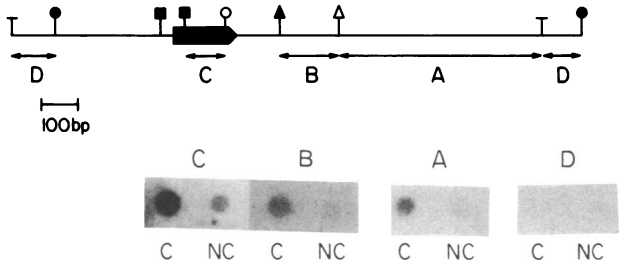

FIG. 1. Transcription of the U1.2 3′ flanking region. Subclones of the coding region and 3′ flanking region were constructed by cloning fragments of the U1.2 repeat unit (7) into M13mp8 and M13mp9. The cloned fragments were as follows: A, *Rsa* I/*Hin*dIII (+250 to + 775 nucleotides 3′ to the U1 RNA); B, *Ava* II/*Rsa* I (+100 to +250); C, positions 30–140 of U1 RNA; D, *Hin*dIII/*Hin*cII (+776 to +884). The coding (C) and noncoding (NC) strands of each fragment were immobilized on Nytran filters and hybridized with [^{32}P]RNA synthesized in nuclei from hatched blastula embryos. The filters were washed and the bound RNA was detected by autoradiography. bp, Base pairs.

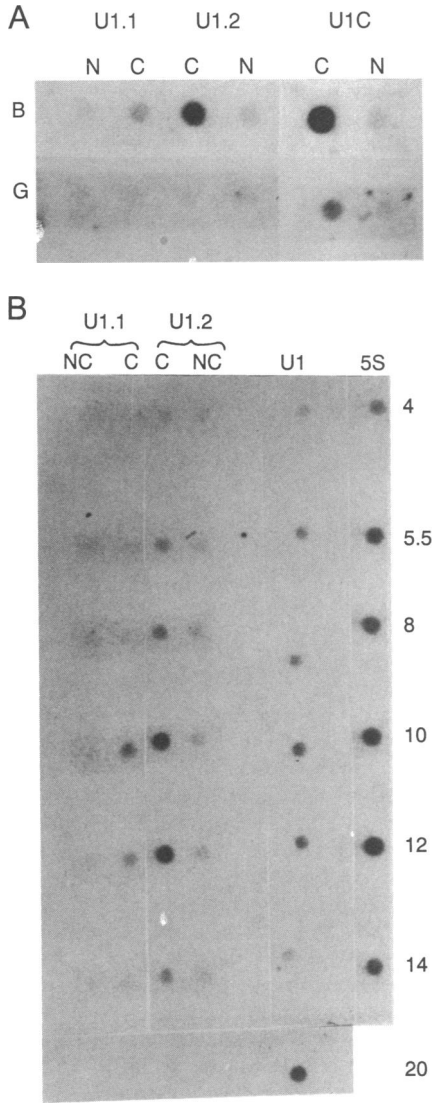

FIG. 2. Transcription of the U1 and 5S genes during development. (*A*) Subclones from the 3′ end of the U1.1 (+68 to +240) and U1.2 (+100 to +250) and the coding region (positions 30–140) were hybridized with 2×10^6 cpm of RNA synthesized in hatched blastula (B) or gastrula (G) nuclei. The filters were washed and the bound RNA was detected by autoradiography. (*B*) RNA synthesized in nuclei from embryos 4, 5.5, 8, 10, 12, 14, and 20 hr after fertilization was hybridized to the U1.1 and U1.2 gene-specific probes. Equal amounts of RNA (10^6 cpm) were used as input from 4–14 hr and 10^7 cpm was used as input for the gastrula (20 hr) RNA. In a separate hybridization, 2×10^5 cpm of RNA (4–14 hr) or 2×10^6 cpm of RNA (20 hr) was hybridized to the U1 and 5S coding region DNAs. In the 20-hr sample the 5S coding region DNA was omitted. The filters were washed and the bound RNA was detected by autoradiography.

Transcription Extends into the 3′ Flanking Region. The subclones from the 3′ flanking region of pU1.2 (Fig. 1) were

hybridized to RNA synthesized in isolated nuclei from hatching blastula embryos and the hybrids were detected by autoradiography (Fig. 1). There was transcription of only the coding strand of the DNA. The B and A subclones, but not the D subclone, were transcribed, suggesting that transcription terminates *in vitro* prior to the D subclone. As judged by the relative intensity of the hybridization, there was a decrease in the extent of transcription in the large A subclone, relative to the smaller B subclone, suggesting that transcription terminates within this subclone. There is not equimolar transcription of the flanking regions relative to the coding region. This could be due both to the activity of other U1 genes and to the fact that probably only a portion of the transcripts extends 3′ to the gene. We have assumed that the proportion of "read-through" transcripts is constant at different developmental stages. This assumption is supported by the constant proportion of read-through transcripts observed between 4 and 12 hr after fertilization (Figs. 2*B* and 3), a time when the major tandemly repeated genes are very active. Thus, measurement of the hybridization to the first portion of the 3′ flanking region should be a measure of the relative activity of the U1.2 gene. A probe from a similar region of the U1.1 gene (7) was used to measure the expression of the U1.1 genes, which are expressed about 5 times less than the U1.2 genes, in agreement with their lower gene copy number (2, 7).

Transcription of the Repeated U1 Genes During Development. In an initial experiment, nuclei were prepared from hatching blastula embryos and from gastrula embryos. RNA was synthesized *in vitro* and hybridized to the two gene-specific probes as well as to the U1 coding region probe. The resulting autoradiograms are shown in Fig. 2*A*. There was no detectable transcription of the 3′ flanking region of either the pU1.1 or pU1.2 gene in nuclei from gastrula embryos, although there was still synthesis of RNA that hybridized with the U1 coding region (Fig. 2*A*).

To investigate the time course of expression of the repeated U1 genes during development, nuclei were isolated from morula (4 hr after fertilization), early blastula (5.5 hr), hatched blastula (8 hr), 10-hr, 12-hr, 14-hr, and gastrula (20 hr) embryos. Mesenchyme blastula was at 14 hr after fertilization and gastrulation was complete by 20 hr. The nuclei were incubated with radiolabeled UTP and the RNA was hybridized to the repeat specific and coding region probes for U1 and 5S RNA. The autoradiograph for one complete series is shown in Fig. 2*B*. The rate of 5S gene transcription was also measured on the same RNA samples. The RNA was hybridized to the U1 3′-specific probes and the coding regions for 5S and U1 RNA in separate bags to eliminate the possibility of competition between the two probes for the same RNA molecule. To ensure that the efficiency of hybridization was similar in each hybridization, a known amount of [^{3}H]RNA (containing both U1 and 5S RNA) was included in every hybridization as a control for hybridization efficiency. The hybridization efficiency was constant in all samples. To confirm the lack of expression of the U1.2 genes, 10 times as much RNA (cpm) was used in the hybridization at the gastrula (20 hr) stage. Even with a much stronger signal for the total U1 synthesis, there was no detectable expression of the tandemly repeated genes.

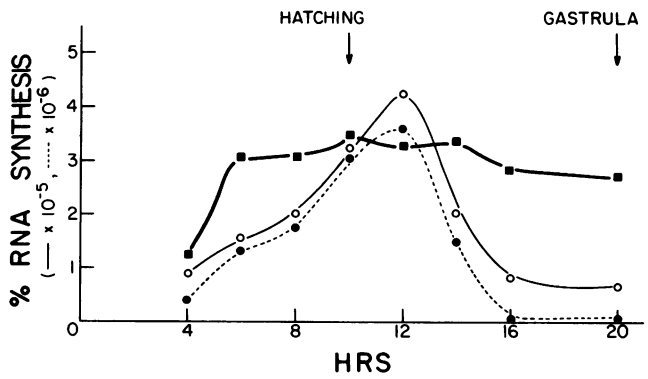

FIG. 3. Expression of the 5S and U1 genes during development. The amount of transcription of the U1.2, total U1 RNA, and 5S rRNA was determined by scanning the dots similar to Fig. 3 with a densitometer and by determining the bound radioactivity by liquid scintillation counting. The results are expressed as a proportion of total RNA synthesis. The results are the average of three independent experiments. ○, Total U1 transcription; ●, U1.2 transcription; ■, 5S rRNA transcription.

The results were quantitated by counting the "dots" in a liquid scintillation counter and by densitometry. Similar results were obtained by both methods. The rates of transcription of all the U1 genes, the 5S gene, and the U1.2 gene during development from three independent experiments were averaged and are plotted in Fig. 3. There was a decrease by a factor of 8 in the relative rate of total U1 RNA transcription between the blastula and the gastrula stage. However, there was no detectable transcription of the U1.1 or U1.2 genes in gastrula nuclei. As a control, nuclear RNA was also hybridized to 5S rRNA gene coding dots. Nijhawan and Marzluff (4) have shown that 5S rRNA synthesis increases from cleavage to blastula and then remains relatively constant. Our results show that the relative rate (percent of total RNA synthesized) of 5S rRNA synthesis increases 4- to 5-fold between morula and early blastula and then remains constant. The relative rates of synthesis of both 5S and total U1 RNA agree well with our previous estimates obtained from pulse labels *in vivo* (4). These results suggest that the expression of the tandemly repeated U1 RNA genes is greatly reduced by 6 hr after hatching and is not detectable in the gastrula stage (Figs. 2 and 3), suggesting that these genes are inactivated at about the mesenchyme blastula stage.

Different U1 RNAs Are Present in Early and Late Embryo Nuclei. Previously we had shown that the U1 RNA found in sea urchin eggs has the same sequence as the U1 RNA expressed from the tandemly repeated gene set (7). The sequence of U1 RNA from gastrula nuclei indicated that there was a heterogeneity at nucleotide 122, suggesting that gastrula nuclei contained a mixture of U1 RNAs (see ref. 7; Fig. 4). To examine this possibility more definitively, we synthesized an oligonucleotide complementary to nucleotides 147–125 of U1 RNA with the 3′ end just prior to the site of heterogeneity. This oligonucleotide was labeled at the 5′ end with polynucleotide kinase, hybridized with nuclear RNA, and then extended with reverse transcriptase using three deoxynucleotide triphosphates and one dideoxynucleotide triphosphate as substrates. The transcripts in each reaction stop at the first nucleotide encountered opposite which the dideoxynucleotide is incorporated. As shown in Fig. 4, there is a difference at nucleotide 122 between the major U1 RNA in morula and pluteus nuclei. In morula, the predominant RNA has a C at nucleotide 122, while in pluteus the majority of the RNA has a U at nucleotide 122. This is evidenced by the changes in both the G and A lanes. The sequence of the major U1 RNA in morula nuclei is that predicted from the tandemly repeated genes (2, 6, 7), and the sequence of the U1 RNA isolated from pluteus nuclei is consistent with the heterogeneity we reported (7). Since at least some of the U1 RNAs synthesized in morula and blastula embryos persist in the pluteus embryo (4), it is not surprising that both U1 RNAs are present in the pluteus embryo. After inactivation of the tandemly repeated gene set, there is accumulation of the "late" U1 RNA at a slow rate due to the decline in total U1 RNA synthesis (ref. 4; see Fig. 2*B*). Because of the stability of U1 RNA, this will alter the ratio of the two U1 RNAs relatively slowly during development.

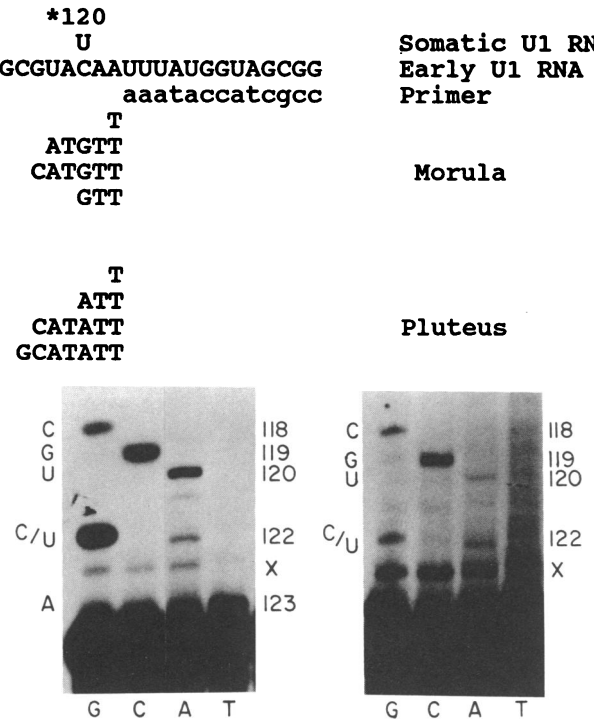

FIG. 4. Different U1 RNAs are present in morula and pluteus nuclei. (*Upper*) The assay is shown schematically, with the U1 RNA sequence around nucleotide 120 in capital letters and the primer in lowercase letters. Below are the predicted extension products from the U1 repeat (morula, C at position 122) and from the postulated constitutive gene (pluteus, U at position 122). (*Lower*) Nuclear RNA from morula (M) and pluteus (P) nuclei was hybridized to an oligonucleotide complementary to nucleotides 137–124 of U1 RNA. The oligonucleotide was 5′-end-labeled and extended with three deoxynucleotide triphosphates and one dideoxynucleotide triphosphate. The ddNTP used is indicated below each lane. The sequence of the RNA is given next to the autoradiogram. In the morula sample most of the U1 RNA has a C at position 122, and in the pluteus sample most of the U1 RNA has a U at position 122. Band X is a background fragment that was observed in all lanes. The A at position 123 is visible in the morula sample but was obscured by the labeled primer in the pluteus sample.

The repeated genes we have isolated are the major U1 RNA genes in the sea urchin (2, 5), and the other gene(s) expressed in later embryonic stages must be previously undetected genes. Since there is also some RNA with a U at nucleotide 122 in the morula embryo, it is likely that these previously undetected genes are expressed constitutively.

DISCUSSION

During early development there is a programmed temporal pattern of gene activity. The necessary gene products for synthesis of most mRNAs are the snRNAs, which are required for RNA processing. In sea urchin embryos, there is a high rate of synthesis of snRNAs starting early in embryonic development (4). In addition, there are a large amount of snRNAs necessary for the processing of the maternal mRNAs during oogenesis. The large number of tandemly repeated genes encoding U1 RNAs is necessary for the synthesis of the large amount of U1 RNA early in sea urchin oogenesis (3). The data presented here suggest that they are also active during early embryogenesis, another time when there is very active synthesis of U1 RNA (4).

In these experiments, we have measured the relative rate of U1 RNA synthesis (fraction of total RNA synthesized) at different developmental stages. Since there is a decrease in the absolute rate of synthesis of RNA on a per nucleus basis during development (22), and a reduced rate of transcription in isolated nuclei from later developmental stages (14), our data overestimate the rate of transcription at later (gastrula) stages. Thus, in reality, the decrease in absolute transcription rate of the U1 genes expressed early in development is greater than suggested here. This does not alter the major conclusion that these U1 genes are inactivated between the mesenchyme blastula and gastrula stages. After hatching, both the absolute and relative rate of U1 RNA synthesis drops dramatically, in agreement with our previous measurements with pulse-labeled RNA (4).

The results presented here suggest that the drop in rate of synthesis may be due to inactivation of at least the majority of the members of the tandemly repeated gene set. The U1 gene(s) active later in embryogenesis encodes a U1 RNA that differs in at least one nucleotide from the U1 RNA present in eggs and expressed in morula embryos. This U1 gene could be derived from a subset of U1 genes linked to the tandemly repeated genes that remain active. More likely, there is another set of U1 genes, which is not linked to the tandemly repeated genes and which are present in low copy number, a situation similar to that of the histone genes. The much lower synthesis rate of U1 RNA required after mesenchyme blastula would not require a large number of copies of U1 genes. The high copy number genes would be required for synthesis of a large amount of U1 RNAs in the oocyte (3) and egg (2), as well as for the rapid rate of synthesis in morula and early blastula (4). Since this "late" U1 RNA is also present in small amounts in morula embryos, it is likely that the late gene set is expressed constitutively.

The drop in the rate of synthesis of total U1 RNA synthesis may be due to inactivation of the early gene set, while the late gene set continues to be active. Until the late genes have been isolated, it is not possible to determine whether they are physically linked to the early genes and whether their rate of expression varies during embryogenesis.

There is a similar switch in expression of histone genes during sea urchin oogenesis and embryogenesis (23). The tandemly repeated set of α-histone genes is expressed at high rates early in embryogenesis and is inactivated between blastula and gastrula (10–12). There is constitutive expression of the late histone genes during early development and then an increase in expression after blastula at a time when the expression of the early genes has declined (12, 24). The net result is a great reduction in the synthesis of total histone mRNA coupled with a change in the histone mRNAs expressed, a situation similar to that seen with U1 RNA. The α-histone genes of *L. variegatus* are apparently shut off much later than the α-histone genes in *Strongylocentrotus purpuratus* and *Lytechinus pictus*, as judged by the lack of detectable late histone proteins until after the mesenchyme blastula stage (25), at about the same time as the *L. variegatus* U1 genes are inactivated. It is possible that the α-histone and U1 tandem repeats are inactivated at the same time.

In both *Xenopus* and mouse embryos, there are changes in the U1 RNAs during early embryogenesis. In *Xenopus*, there is rapid synthesis of U1 RNAs from a tandemly repeated gene set (26, 27) at the time of the initial activation of RNA synthesis (28, 29). There is subsequent synthesis of a different U1 RNA later in development (30). There are similar variations in the U4 RNAs synthesized during early *Xenopus* development (30). In mice, there is synthesis of two U1 variants, U1a and U1b, starting between the two- and the eight-cell stage (31). Both these U1 variants continue to be synthesized during embryogenesis, although only U1a RNA is expressed in most terminally differentiated cells (32). There is no known functional difference between the different U1 RNAs in any species. It is possible that the changes in U1 RNA during development are simply due to switches in the genes utilized, with the nucleotide changes reflecting neutral

changes in the U1 snRNA structure. It is also possible that there are subtle differences in the function of the different U1 RNAs that are important for the proper developmental patterns of gene expression.

The pathway of U1 RNA (and snRNA biosynthesis in general) is not well understood. The existence of developmentally regulated snRNA genes should allow the determination of sequences involved in U1 snRNA expression as well as sequences required for the developmental regulation of expression.

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