Translational Regulation of Histone Synthesis in the Sea Urchin *Strongylocentrotus purpuratus*

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ABSTRACT The pattern and schedule of histone synthesis in unfertilized eggs and early embryos of the sea urchin *Strongylocentrotus purpuratus* were studied using two-dimensional gel electrophoresis. After fertilization there is an abrupt change in the pattern of histone variant synthesis. Although both cleavage-stage- and α-histone mRNA are stored in sea urchin eggs, unfertilized eggs synthesize only cleavage-stage (CS) variants. However, after fertilization, both CS and α messages are translated. Since α histone mRNA isolated from unfertilized eggs can be translated in vitro, the synthesis of α histone subtypes appears to be under translational control. Although the synthesis of α subtypes is shown here to occur before the second S phase after fertilization, little or no α histone is incorporated into chromatin at this time. Thus, early chromatin is composed predominantly of CS variants probably recruited for the most part from the large pool of CS histones stored in the unfertilized egg.

During sea urchin development, histone variants of the H1, H2A, and H2B classes appear sequentially in the chromatin (1–6). Variants of each class are coded by different members of the histone multigene family and differ from each other in primary amino acid sequence (5, 7–9). Characteristic sets of histones are made and assembled into chromatin at each of several developmental stages. Cleavage-stage (CS) histones are the first to appear (4). They are presumably made during oogenesis and are stored in the unfertilized egg in large quantities (at least several hundred haploid DNA equivalents [10, 11]). The CS variants participate in an extensive remodeling of the sperm chromatin after fertilization, which results in a reduction in the nucleosome repeat length (12). Almost immediately after fertilization, the sperm H1 variant is replaced by CS H1. Concomitant with DNA synthesis, substantial amounts of CS H2A and CS H2B accumulate in the chromatin. These protein transitions can take place in the absence of protein synthesis. Later in development the α variants appear, and, by the morula stage, chromatin is composed predominantly of nucleosomes that contain α histones (1, 4). Synthesis of late histones (designated β, γ, and δ) is initiated at the mesenchyme blastula stage (1, 4). When sea urchin maternal mRNA is translated in vitro, both α (6, 8, 13) and CS histones (8) are made. Because both α and CS histone mRNAs are stored in the sea urchin egg, it is surprising that mature eggs contain only CS proteins. There is no detectable storage of α histones (11). These observations suggest that the synthesis of α histone subtypes may be under translational control. On the basis of the observation that newly synthesized α variants, incorporated into chromatin, are first detected at about the third S phase after fertilization, others have suggested a qualitative translational control for histone variant synthesis (4, 6).

Fertilization of the sea urchin egg stimulates the rate of protein synthesis ~15-fold by 2 h postfertilization (14). This is accompanied by an ~30-fold increase in the fraction of ribosomes found in polysomes but not by large changes in the efficiency of translation (defined as the rate of protein synthesis/polysomal mRNA/time) (15). The increase is almost entirely dependent on maternal message since it occurs in actinomycin D-treated embryos (16) and enucleated egg fragments (17). These results taken together suggest a recruitment of untranslated maternal message into polysomes after fertilization. At 2 h after fertilization 90% of the polysomal mRNA is maternal, whereas by gastrula stage virtually all is newly transcribed from embryonic genes (15). Using an isoelectric focusing gel system that does not resolve basic proteins, Brandhorst could detect few obvious differences in the protein species synthesized before and after fertilization (18). Therefore, it was concluded that there are few classes of abundant messages translated after fertilization that are not already being translated and that the major increase in protein synthesis rate that accompanies fertilization is not accomplished by the recruitment of qualitatively new messages but rather by the translation of more mRNAs of the same types (18). However, there is an increasing amount of evidence that suggests that histone mRNAs behave differently than much of the maternal message. Wells et al. (19) have shown that, unlike most messages, the recruitment of H3 mRNA onto polysomes is delayed. Venezsky et al. (20) by *in situ* hybridization, showed that RNA transcripts complementary to a genomic histone repeat are

THE JOURNAL OF CELL BIOLOGY · VOLUME 94 JULY 1982 219–223
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found in high concentration in sea urchin egg pronuclei. However, a similar high nuclear concentration is not observed for two other abundant maternal mRNAs.

Although histone mRNA is an abundant maternal messenger class (~3 pg/egg), amounting to possibly as much as 8% of the mRNA pool (21), a detailed analysis of histone variant types synthesized before and after fertilization has not been made. In this paper we use a polyacrylamide gel system that is specifically designed to resolve histone variants (22) and to separate them from contaminating proteins (23) in order to examine the pattern of histone synthesis in acid extracts of unfertilized and fertilized eggs. We find that (*a*) before fertilization CS histones are the only type of histone made, (*b*) after fertilization or artificial activation both CS and α variants are synthesized, and (*c*) α synthesis begins before the second S phase, which is earlier than other reports have indicated.

MATERIALS AND METHODS

Strongylocentrotus purpuratus were obtained from Pacific Bio-marine Laboratories Inc., Venice, CA. Urchins were maintained in a refrigerated aquarium and fed laminaria collected at the Marine Biological Laboratory, Woods Hole, MA.

For culture and labeling of eggs and embryos, *S. purpuratus* eggs were collected after intercoelomic injection of 0.5 M KCl, allowed to settle, filtered through 202-μm Nitex mesh, and washed three times in fresh Millipore-filtered seawater (Instant Ocean, Eastlake, OH) containing 10 μg/ml gentamycin (Sigma Chemical Co., St. Louis, MO) and 5 mg/ml streptomycin (Sigma Chemical Co.). Eggs were incubated with antibiotics for 1.5 h before the addition of isotope. Eggs were then cultured in 50 vol of Millipore-filtered (Millipore Corp., Bedford, MA) seawater (MPFSW) containing 100 μCi/ml [^{3}H]lysine (L-[4,5-^{3}H(*N*)]lysine [New England Nuclear, Boston, MA]; 64.5 Ci/mmol) in a conical 1.5-ml Eppendorf tube (Brinkman Instruments, Inc., Westbury, NY) that was rotated at 30 rpm at 15°C for the times specified in the figure legends. To each 1 ml of culture, 25 μl of 1 M HEPES pH 7.3, dissolved in MPFSW, was added to buffer the 0.01 N HCl in which the isotype is packaged. More than 90% of the eggs could be fertilized after the incubations.

Monospermic embryos were cultured as previously described (24) with 50 μCi/ml of [^{3}H]lysine for the periods specified in the figure legends.

Eggs were artificially activated with N-9 seawater, which was prepared by adjusting ordinary seawater to pH 9.0 with NH_4OH (25). In these experiments, eggs were first cultured with 100 μCi/ml [^{3}H]lysine in N-9 seawater for 1 h. At the end of this time, eggs were washed twice with MPFSW and then cultured in ordinary MPFSW containing this same amount of isotope, for the periods given in the figure legends.

To produce actinomycin D–treated blastulae, eggs were incubated in 25 μg/ml actinomycin D (grade 1; Sigma Chemical Co.) dissolved in MPFSW for 90 min before fertilization. Each lot of actinomycin D was previously tested. Eggs were then pelleted by hand centrifugation and fertilized in MPFSW. Fertilized eggs were washed to remove excess sperm, washed twice in 25 μg/ml actinomycin D in MPFSW, and cultured in this solution in the dark to the blastula stage (5, 26, 27).

Nuclei from blastulae grown in actinomycin were isolated by Dounce homogenization in 0.075 M NaCl, 25 mM Tris, 1 mM EDTA pH 7.6 solution containing 0.01% Triton X-100 as described in Poccia et al. (24).

Polyspermic fertilization was done as previously described (11). The degree of polyspermy was determined in eggs fixed in Carnoy's solution (ethanol:acetic acid 3:1). An aliquot of fixed eggs was dried on microscope slides, and nuclei were stained with 1% orcein in 75% acetic acid. The number of male pronuclei was counted under bright-field optics equipped with a green filter.

Nuclei were prepared as previously described (25) by lysis of eggs in a solution of 15% ethanol, 0.2% Triton X-100 (Triton), 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) by gentle shaking.

Two-dimensional gel electrophoresis was performed according to Savic and Poccia (23) with the modifications described by Poccia et al. (10). The first dimension is based on Triton gel systems previously described (22). Briefly, the 6-cm first dimension resolving gel was 6 M urea, 6 mM Triton, 5% acetic acid, 12% acrylamide, 0.8% *bis*-acrylamide. The riboflavin-polymerized 1-cm stacking gel was 5.2 M urea, 9 mM potassium acetate, pH 4.5, 4.8% acrylamide, 0.032% bisacrylamide. The second dimension was a 6-cm-long (1-mm-thick) SDS slab gel run as described by Laemmli (28), with the amount of *bis* in the acrylamide stock halved.

Gels were stained with 0.3% Coomassie Blue in 50% MeOH, 7.5% acetic acid, destained in 50% MeOH, 7.5% acetic acid, and then fluorographed (29, 30). Preflashed Kodak XAR-5 x-ray film was used.

For acid extraction, both eggs and embryos were washed free of exogenous label, using gentle hand centrifugation (~1,000 rpm for 10 s) with cold MPFSW. Eggs were disrupted by the addition of 0.075 M NaCl, 0.025 M EDTA, 0.01 M Tris pH 8.0, 0.1% Triton, 0.1 mM PMSF, 10 mM DTT, followed by Dounce homogenization and then extracted with an equal volume of 0.8 N H_2SO_4 at 0°C for 8 h, with occasional vortexing. All subsequent steps were done in 1.5-ml Eppendorf test tubes. The extract was centrifuged at 10,000 *g* for 30 min to remove acid-insoluble material and the supernatant was made 20% in trichloroacetic acid (TCA) by adding 100% (wt/vol) TCA solution. The pellet was reextracted with 0.4 N sulfuric acid and centrifuged. This supernatant was made 20% in TCA and pooled with the first extract. After 30 min on ice, the precipitate was pelleted, washed twice with acetone, 0.1% H_2SO_4 at −20°C, and washed several times with cold acetone. The protein pellet was dried at room temperature *in vacuo* before resuspension in sample buffer. Nuclei were twice acid-extracted in 0.4 N H_2SO_4, and the combined supernatants were processed in the same way described above for the acid extracts.

RESULTS AND DISCUSSION

Electrophoretic Analysis of CS and α Variants

To analyze histone synthetic patterns in whole eggs and embryos, a high-resolution electrophoretic system is required. A system has been described (23) that separates H1 and core histone variants on the same slab, even when large amounts of contaminating basic proteins are present in the sample. Electrophoresis on Triton/6 M urea gels in the first dimension results in preferential retardation of the histones (22), which then migrate off the diagonal (away from contaminating ribosomal proteins) upon SDS gel electrophoresis in the second dimension.

Figure 1*A* shows a Coomassie Blue–stained gel of the histones that are incorporated into sea urchin blastulae grown continuously in the presence of 25 μg/ml actinomycin D from 90 min before fertilization. Both CS and α histones are present. These histones originate from translation of stored CS and α mRNA (4) and from stored CS histones (10). Storage of histone mRNA has also been demonstrated by translation of maternal mRNA in the wheat-germ cell-free synthesis system (6, 8, 13, 31).

Figure 1*B* shows a Coomassie Blue–stained gel of acid-soluble protein extracted from isolated male pronuclei. As previously documented, male pronuclear chromatin at 70 min postfertilization contains CS H1 and no sperm H1; CS H2A and sperm H2A but no α H2A; CS H2P and proteins O and P which are modifications of or substitutes for sperm H2P; H3 and H4 species; and proteins M and N (10). M is probably the H2A variant Y6 of Newrock et al. (5) and N may be an H1 protein that runs between sperm H1 and α H1 on this gel system. The relative positions of O, P, and H4 are variable from gel to gel and are checked against internal standards run in parallel (10).

The Pattern of Protein Synthesis Changes after Fertilization

The standards shown in Fig. 1 were used to identify histone variants synthesized in the unfertilized sea urchin egg. Acid-soluble proteins labeled with [^{3}H]lysine were extracted from unfertilized eggs and electrophoresed. A fluorogram of the gel is shown in Fig. 2*A*. The CS H1, CS H2A, and CS H2B are labeled. Even in overexposed gels, no α variants are present. In addition to bands that comigrate with the CS proteins, we see proteins whose mobilities correspond to O, P, and M described above. All of these newly synthesized proteins are soluble in 0.4 N H_2SO_4, all contain lysine, and all comigrate with known standards, but additional protein chemistry will be necessary

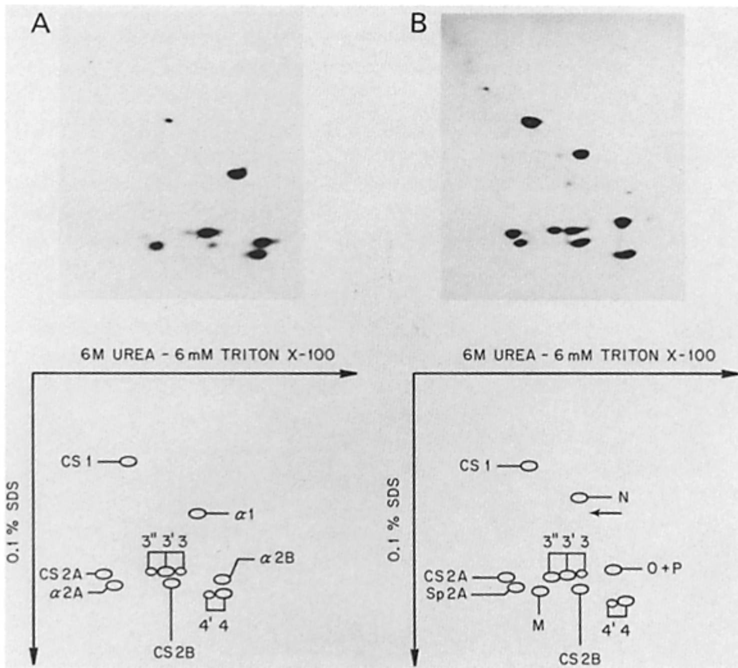

FIGURE 1 Electrophoretic separation of protein standards. (*A*) Histones isolated from chromatin of blastulae grown in the presence of 25 μg/ml actinomycin D. (*B*) Histones from male pronuclei. Pronuclei were isolated 70 min after fertilization. Proteins were stained with Coomassie Blue. The degree of polyspermy was ~30 pronuclei/egg. Arrow indicates where sperm H1 would migrate.

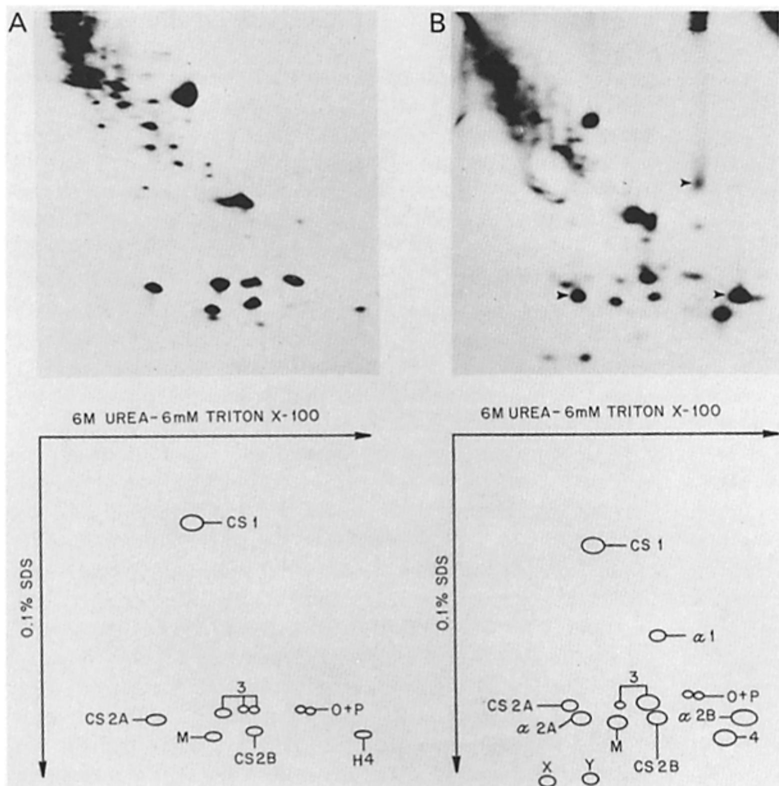

FIGURE 2 Fluorographic comparison of the proteins synthesized by unfertilized and fertilized eggs: (*A*) 2-h labeling of unfertilized eggs, (*B*) 5- to 70-min postfertilization labeling. Each was labeled with 100 μCi/ml [^{3}H]lysine. Lettering indicates comigration with well-characterized standards and is not proof of protein identity. The identity of low molecular weight spots designated *X* and *Y* is not known. Arrowheads point to newly synthesized α histones.

to confirm their identity. Fig. 3 shows that the unfertilized eggs make the same proteins when the labeling time is of different lengths. Fig. 3 *A* is a 2-h labeling and Fig. 3 *B* is a 4-h labeling pattern.

Using one-dimensional SDS gels, Ruderman and Schmidt (32) showed the unfertilized egg to be active in histone synthesis. However, since both CS- and α-histone mRNAs are present in the egg, it was uncertain just which of the histone types was synthesized. Our data show that only CS mRNAs are translated by the unfertilized egg; α proteins either are not synthesized or are made in insignificant amounts. These data are consistent with the observation that several hundred haploid equivalents of CS proteins are stored in the urchin egg (11), whereas no α storage is detected. If α histones were synthesized at levels comparable to CS variants in the unfertilized egg, α histones would have to be preferentially degraded.

If α histones are not made before fertilization, when is α mRNA first translated? Earlier reports show that α proteins are the predominant histone variants made and incorporated into chromatin at the third S phase after fertilization (4). However,

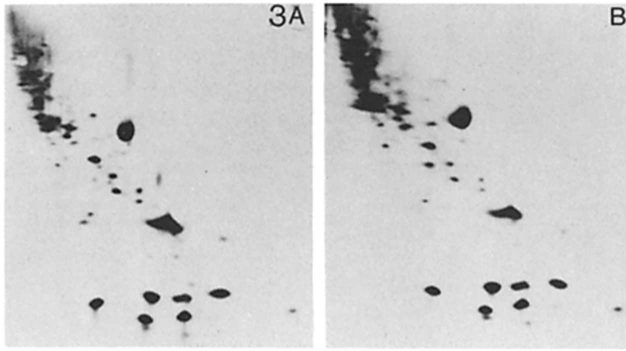

FIGURE 3 Fluorograph of unfertilized egg proteins labeled in vivo with 100 μCi/ml [^{3}H]lysine. (*A*) 4-h labeling, (*B*) 2-h labeling.

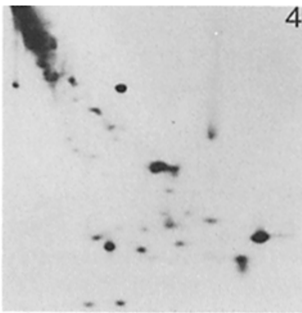

FIGURE 4 Fluorograph of proteins synthesized by artificially activated eggs. Eggs were first cultured for 1 h in pH 9.0 NH_3-seawater containing 100 μCi/ml [^{3}H]lysine. Eggs were washed and then cultured for an additional hour in ordinary MPFSW containing 100 μCi/ml [^{3}H]lysine. Proteins were acid extracted and separated using the gel system described in Materials and Methods.

using an isotope of higher specific activity, the two-dimensional gel system, and whole cell extracts, we reexamined the time after fertilization when α synthesis begins.

In *S. purpuratus* grown at 15°C, the first S period is at 30–45 min postfertilization, the second S period at 105–125 min, the first cell division at 100–120 min, and the third S period at 170–185 min (33). Fig. 2*B* shows histones labeled with [^{3}H]lysine continuously from 5–70 min postfertilization (30 min before second S phase). A striking change is apparent. In addition to the continued synthesis of CS proteins, α mRNA translation has commenced. In the H2A class, the α H2A becomes the predominant variant synthesized. In the H2B class, α H2B is the major protein. The rapidity of the change suggests that the appearance of α is not due to the translation of a small amount of newly synthesized RNA (34). Fig. 4 shows that the CS to α transition can also be triggered by artificial activation with pH 9.0 ammonia-seawater (26). The pattern of proteins made is very similar to that seen after fertilization. Thus there is no apparent role of the sperm in control of the histone synthetic switch that cannot be mimicked chemically. Preliminary results show that this early appearance of α histones occurs even in the presence of actinomycin D (data not shown).

α Histones Are Not a Major Component of Male Pronuclear Chromatin

The synthesis of histones well before their incorporation into replicating chromatin is unlike the situation reported for many other cell types, in which histone and DNA synthesis are more or less tightly coupled within a given cell cycle and nonchromosomal histone pools are believed to be small or nonexistent (35). The oocytes and early embryos of sea urchins and frogs differ in that histone and DNA synthesis are uncoupled and substantial pools are maintained (10, 11, 36–39).

Although synthesis of the α variants occurs within the first cell cycle, there is no detectable incorporation of α histones into the male pronuclear chromatin during this time, as judged by Coomassie Blue staining (Fig. 1*B* and Poccia et al. [10]). However, later in the morula stage, α becomes the predominant histone variant type in the chromatin (1). Since there are enough CS histones stored in the unfertilized egg to package all the DNA made up to at least the blastula stage, but blastula chromatin contains largely α variants, at some point in development α incorporation into chromatin must predominate over CS incorporation.

It is possible that the flow of α histones, newly synthesized affter fertilization, through a large pool of mostly preformed CS histones, results in a substantial dilution effect. This would delay the appearance of significant levels of α in the chromatin until after the first few cell cycles, when the composition of the pool may change, reflecting greater input of α forms and perhaps withdrawal of CS forms. Although sea urchin histones, once they are incorporated into chromatin, are long-lived (2, 40), nothing is known about turnover of the stored histone pool.

A second possibility for the delay between α histone synthesis and appearance in the chromatin is that α variants, once made, are sequestered until the third S phase when they are released and assembled into chromatin. Thus chromatin in the third cycle would undergo a qualitative, rather than a simple quantitative transition in its composition (cf. reference 4).

To clarify these issues, it is essential to compare, at several developmental stages, the histone composition of the chromatin with its cognate histone pool. Clearly, further study on the fate of the stored CS proteins is necessary to determine whether they are degraded, rendered assembly incompetent, or assembled into chromatin at a later developmental stage. In some earlier papers (4, 11) labeled α histones can be seen in extracts of nuclei isolated at the two-cell stage. However, no careful quantitative measurements of the ratio of α to CS variants in embryonic nuclei of the first three or four cycles after fertilization are yet available. Such ratio measurements are hampered by contamination with nonchromosomal proteins (including histones from the pool) and low nuclear yields.

There are many possible mechanisms for the translational regulation of α histone mRNA. An old idea is that specific proteins might "mask" the mRNAs in the unfertilized egg. At fertilization or later in development there would be a selective unmasking dictated by the maternal program. Recent work, however, suggests another possible mechanism. A significant amount of histone gene transcripts, at least 12%, is sequestered in the female pronucleus and is later released into the cytoplasm (20). The observed delayed recruitment of H3 histone message (19) is consistent with this observation. It is likely, in light of our results, that much of histone mRNA recruitment is of α mRNA. We are now testing these ideas. If α message is sequestered in the female pronucleus, we would expect that the message isolated from nucleated egg halves would code for both α and CS histones, while mRNA from annucleate merogons would only code for CS variants.

L. Herlands would like to thank Joel Rosenbaum for his guidance.

This work is supported in part by National Institutes of Health grants GM17383 and HD12982 and the Harry Winston Foundation.

Received for publication 12 February 1982, and in revised form 11 March 1982.

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