

Electrophoretic Analysis of the Stored Histone Pool in Unfertilized Sea Urchin Eggs: Quantification and Identification by Antibody Binding

J. SALIK, L. HERLANDS, H. P. HOFFMANN, and D. POCCIA

Department of Biology, Amherst College, Amherst, Massachusetts 01002; Department of Biology, State University of New York at Stony Brook, Stony Brook, New York 11794; and The Rockefeller University, New York, New York 10021

ABSTRACT A maternal store of histones in unfertilized sea urchin eggs is demonstrated by two independent criteria. Stored histones are identified by their ability to assemble into chromatin of male pronuclei of fertilized sea urchin eggs in the absence of protein synthesis, suggesting a minimum of at least 25 haploid equivalents for each histone present and functional in the unfertilized egg. In addition, electrophoretic analysis of proteins from acid extracts of unfertilized whole eggs and enucleated merogons reveals protein spots comigrating with cleavage stage histone standards, though not with other histone variants found in later sea urchin development or in sperm. Quantification of the amount of protein per histone spot yields an estimate of several hundred haploid DNA equivalents per egg of stored histone. The identity of some of the putative histones was verified by a highly sensitive immunological technique, involving electrophoretic transfer of proteins from the two-dimensional polyacrylamide gels to nitrocellulose filters. Proteins in amounts $<2 \times 10^{-4} \mu\text{g}$ can be detected by this method.

The early development of the sea urchin embryo exhibits characteristically short cell cycles and rapid rates of DNA replication as the zygote gives rise to a multicellular morula. Sufficient quantities of histones must be available for packaging newly synthesized DNA.

DNA synthesis in the early sea urchin embryo is among the most rapid known for a eukaryote, the S phase of the early cell cycles lasting only 11–13 min (1). To accomplish this replication, unfertilized sea urchin eggs possess large stores of DNA polymerase (2, 3, 4) and deoxynucleotide kinases (4), as well as pools of deoxynucleotides (5, 6, 7).

Also stored within the sea urchin egg is a sufficient set of mRNAs to enable virtually normal development of the embryo to the blastula stage, even if embryonic transcription is blocked by actinomycin D (8, 9–13). A major fraction of the maternal mRNAs code for histones and much of the protein synthetic activity of the early embryo is devoted to the production of the basic nuclear proteins (14).

A characteristic of sea urchin development is the sequential appearance in the chromatin of different variants of H1, H2A, and H2B (15–19). The first set of embryonic variants, called the cleavage stage (CS) histones, are synthesized from fertilization to morula, followed by the α variants synthesized from

approximately the eight-cell stage to blastula, and the β , γ , and δ histones synthesized from blastula to pluteus (18, 19). The stored messages used in early histone synthesis code for CS and α variants (11, 13, 20, 21, 22), whereas messages for the β , γ , and δ variants are newly synthesized beginning with the blastula stage (20, 23, 24).

Although generally the timing of histone synthesis is concomitant with DNA synthesis (25), exceptions are known (26, 27, 28). In the *Xenopus laevis* oocyte, a large pool of stored histones has been demonstrated (28). In sea urchins, a pool was inferred by comparison of the specific activities of CS and α variants (19).

We have demonstrated previously that male pronuclear chromatin acquires its usual set of CS histone variants within the first cell cycle after fertilization, even if protein synthesis is blocked (29). In addition, by two-dimensional gel electrophoresis of acid extracts of unfertilized eggs, proteins were found to comigrate with the CS variants (19).

This study was done to obtain a minimum estimate for the amount of stored functional histone present (that is, histone which can be utilized to assemble chromatin as determined by an *in vivo* polyspermic assay), to quantify the amount of each histone class stored in the unfertilized egg by computer scans

of two-dimensional gels, and to further identify the stored histones by immunological procedures.

MATERIALS AND METHODS

Gametes, Fertilization, and Culture of Embryos

Gametes of *Strongylocentrotus purpuratus* were procured and monospermic embryos cultured as previously described (30). Polyspermic fertilization was induced by washing unfertilized eggs twice with 10 vol of 30 mM NH_4Cl in Millipore-filtered seawater (MFSW), adjusted to pH 8 (total wash time, 5–10 min), followed by resuspension of the egg pellet in 25 vol of 0.3% (vol/vol) sperm suspension in normal seawater. 30–60 s after fertilization, as the fertilization membranes appeared, 2 vol of 1 mg/ml mercaptoethylgluconamide (Travenol Laboratories, Los Angeles, Calif.) in calcium-free seawater containing 10 mM EDTA was added to the fertilized egg suspension to facilitate removal of fertilization membranes that otherwise interfere with subsequent nuclear isolations (31). Excess sperm was removed with four washes in MFSW at 15°C. Eggs were cultured as described for monospermic embryos.

The degree of polyspermy was determined in eggs fixed in Carnoy's solution, ethanol:acetic acid (3:1). Fixed eggs were dried on microscope slides and nuclei were stained with 1% orcein in 75% acetic acid. The number of male nuclei per egg was counted under bright-field optics for 250 eggs.

Inhibition of RNA Synthesis

To enrich chromatin for cleavage stage histones (19), eggs were cultured with 25 mg/ml actinomycin D (Sigma grade 1; Sigma Chemical Co., St. Louis, Mo.) in MFSW. Eggs were incubated in actinomycin D for 90 min before fertilization, fertilized, and cultured to blastulae in this solution.

Inhibition of Protein Synthesis

Protein synthesis in polyspermically fertilized eggs was inhibited with 10^{-4} M emetine (32). Emetine was added to the NH_4Cl -MFSW solution used to induce polyspermy and was also present in the seawater for all subsequent washes and culturing.

Protein synthesis was measured as the incorporation of ^{14}C -labeled leucine into trichloroacetic acid (TCA)-precipitable material. Eggs were incubated in NH_4Cl -seawater without emetine, to which 0.5 $\mu\text{Ci/ml}$ ^{14}C -labeled leucine was added. Emetine was added just before fertilization and was present in all subsequent solutions. Control cultures without emetine were run in parallel. After washing away excess sperm, ^{14}C -labeled leucine was added back to the egg cultures and eggs were incubated for 60 min. Aliquots (1 ml) of control and emetine-treated eggs were taken at 15-min intervals up to 60 min. The aliquots were washed in ice-cold seawater, precipitated in cold 10% TCA, collected on Millipore filters with extensive washing, and dried and counted in a liquid scintillation counter. Radioactive incorporation was inhibited to a level >99%, which has been shown to be due to actual synthetic inhibition rather than a change in amino acid pool size or uptake (32).

Nuclear Isolation and Chromatin Purification

Crude nuclei from blastulae, actinomycin D-treated blastulae and polyspermic embryos were prepared according to Poccia et al. (30). Embryos were suspended in 10–20 vol of 0.8 M dextrose and pelleted through 10 ml of 1 M dextrose at 1,000 rpm for 5 min in an IEC PR-1 centrifuge (International Equipment Co., Needham Heights, Mass.). The pellet was resuspended in 30% ethanol, 0.2% Triton X-100 at 4°C, and pelleted at 1,500 rpm for 3 min. This pellet was lysed at 4°C in a solution of 15% ethanol, 0.2% Triton X-100, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF) by gentle shaking. Nuclei were pelleted by centrifuging the lysis mixture at 4,000 rpm for 10 min.

Sperm nuclei were prepared by homogenizing sperm cells in 10% ethanol, 0.2% Triton X-100, 10 mM DTT, and 1 mM PMSF for 2 min at full speed in a Sorvall Omnimixer (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.). Nuclei were pelleted at 5,000 rpm for 5 min.

Crude nuclei were then either extracted directly in 0.4 N H_2SO_4 or purified to chromatin. Chromatin was prepared by washing nuclei once in 0.075 M NaCl, 0.025 M EDTA, 0.01 M Tris, pH 8.0, followed by the three washes in 0.01 M Tris, pH 8.0. The swollen chromatin pellet was resuspended in 0.01 M Tris, pH 8.0, and centrifuged through 1.7 M sucrose, 0.01 M Tris, pH 8.0, at 20,000 rpm for 3 h in a Beckman SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

Histone Isolation

Histones were isolated from crude nuclei or chromatin by extraction overnight at 4°C in 0.4 M H_2SO_4 . The suspension was spun at 12,000 rpm for 10 min. The

supernate was collected and protein precipitated with 20% TCA for 60 min on ice. The proteins were centrifuged for 10 min at 12,000 rpm, washed once with acetone, 0.1% H_2SO_4 , and three times with cold acetone, dried in a desiccator, and stored at -70°C .

Direct Acid Extraction from Whole Eggs

A known volume of eggs, usually 0.5–1 ml, was suspended in 10 ml fixed volume of 0.8 M dextrose. Two 0.1-ml aliquots of this suspension were fixed in ethanol/acetic acid for quantification of the starting number of eggs. The remaining egg suspension was centrifuged through 10 ml of 1 M dextrose at 1,000 rpm for 5 min. The egg pellet was homogenized at 4°C with a loose fitting Dounce homogenizer in 10 vol of distilled water containing 10 mM DTT, 1 mM PMSF, and 0.2% Triton X-100. An equal volume of 0.8 N H_2SO_4 was then added and the homogenate was stirred overnight at 4°C. The suspension was centrifuged at 12,000 rpm for 10 min, the supernate collected, and the acid-soluble protein precipitated with 20% TCA. The precipitate was washed in the cold with acetone, 0.1% H_2SO_4 , followed by three cold acetone washes. The final pellet was dried in a desiccator and stored at -70°C . When ready for use, the entire pellet was solubilized in gel sample buffer (8 M urea, 5% acetic acid, 1% 2-mercaptoethanol, 0.1% crystal violet), and aliquots of this were analyzed by two-dimensional polyacrylamide gel electrophoresis (PAGE).

Quantification of Stored Histones

The starting number of eggs used for acid extraction was determined by the method of Fry and Gross (33). A known aliquot of the starting egg suspension was fixed in Carnoy's fixative, stained with amido black, and resuspended in 5 ml of Carnoy's fixative. Samples of 10, 50, and 100 μl were spread on filters and counted under a dissecting microscope.

The yield of histone recovered from whole-egg acid extraction was estimated to be 40–60% from the percent recovery of trace amounts of ^{14}C -labeled blastula histones added to the initial homogenate.

Gel Electrophoresis

Two-dimensional PAGE were performed according to Savić and Poccia (34) with the modifications of Poccia et al. (29). The first-dimension resolving gel was 6 M urea, 6 mM Triton X-100, 5% acetic acid, 12% acrylamide, 0.08% bis-acrylamide. The stacking gel was 5.2 M urea, 9 mM potassium acetate, pH 4.5, 4.8% acrylamide, 0.032% bis-acrylamide. The second dimension was a slab gel of 15% acrylamide, 0.085% bis-acrylamide, 0.37 M Tris HCl, pH 8.7, 0.1% sodium dodecyl sulfate (SDS) with a stacking gel of 5% acrylamide, 0.13% bis-acrylamide, 0.1% SDS, 0.125 M Tris, pH 6.8.

Preparation of Labeled Histones

Radioactive blastula histones were prepared from blastula cultures grown in the presence of 0.2 $\mu\text{Ci/ml}$ [^3H]lysine and 0.2 $\mu\text{Ci/ml}$ [^3H]arginine from 10 min postinsemination to hatching.

Labeled cleavage stage histones were prepared from two-cell embryos grown in the presence of 0.2 $\mu\text{Ci/ml}$ [^{14}C]lysine and 0.2 $\mu\text{Ci/ml}$ [^{14}C]arginine. Nuclei and histones were isolated as described.

Fluorography

Fluorography was performed by the method of Bonner and Lasky (35) or with Enhance (Bio-Rad Laboratories, Richmond, Calif.). In the first procedure, slab gels were soaked for 3 h in three changes of dimethyl sulfoxide (DMSO), then in DMSO-20% PPO. The gel was then washed with water, dried, and exposed to Kodak X-omat film.

In the second procedure, the gel was soaked for 60 min in Enhance, washed with water for 60 min, dried, and exposed to x-ray film.

Estimates of the amount of histone stored were made by computer scanning of photographs of the gels of acid-extracted egg proteins run in parallel with known quantities of sperm histone samples. Negatives (35mm) were scanned via an Optronics P1000 (Optronics Chelmsford, Mass.) and analyzed using a program developed by Lutin et al. (36). Dye binding was determined to be linear for core histones in the concentration ranges used.

Preparation of Enucleated Egg Fragments

Enucleated merogons were prepared according to the procedure of Krystal and Poccia (37), using a modification of the procedure of Nishioka and Mazia (26). Linear gradients (33 ml) of from 0.325 M sucrose, 67.5% MFSW to 0.92 M sucrose, 7.5% MFSW were constructed. Eggs (0.3 packed mls) in 2 ml of MFSW

were loaded on each gradient. The gradients were centrifuged in a Beckman SW27 rotor at 4°C for 8 min at 7,000 rpm and for 5 min at 12,000 rpm. Enucleated merogons were collected using a syringe with an 18-gauge needle and monitored microscopically for nuclear contamination. Contamination by nucleated merogons was determined to be <0.1%.

Antisera to Histone Fractions H2B and H4

Calf thymus histone H2B and duck red-blood-cell histone H4 were isolated and purified to homogeneity by the method of Westerhuyzen et al. (38). Antisera were prepared by a method similar to that of Stollar and Ward (39) using doses of 200 µg of protein complexed with 100 µg of purified yeast RNA. Rabbits were immunized by intramuscular inoculations of one primary dose emulsified with complete Freund's adjuvant and, two weeks later, of three weekly doses in incomplete Freund's adjuvant. Sera were obtained one week after the third booster.

The antisera were tested by passive hemagglutination assay (40) and the solid phase microradioimmunoassay of Rosenthal et al. (41) using Staphylococcal protein A, which was labeled with ¹²⁵I by the chloramine T method (42). Much of the labeled Protein A was kindly provided by M. Romani of Rockefeller University. Those antisera with high activity and specificity were selected for use in immunofluorography of electrophoretic transfers.

Immunofluorography

Polyacrylamide slab gels were blotted and electrophoretically transferred to nitrocellulose membrane filters (Millipore HA, 0.45 µm) by the procedure of Towbin et al. (43) in a gel destaining apparatus (E-C Apparatus Corp., St. Petersburg, Fla.). The unstained electrophoretic blots were gently agitated in 2.5% bovine serum albumin (BSA) in slightly modified Denhardt's solution (0.02% polyvinylpyrrolidone, 0.08% Ficoll, 2.0% glycine, 0.1 M NaCl, 1.0 mM EDTA, pH 7.0, and 0.02% sodium azide, 0.01 M sodium citrate) for 2 h at room temperature (44). The absorbed sheets were incubated in antisera diluted (1:50 or 1:100) with Denhardt's BSA solution for ~16 h, rinsed with a gentle stream of saline solution, and labeled with [¹²⁵I]protein A in Denhardt's BSA solution for 1 h. The applied radioactivity was usually 1 × 10⁶ cpm per sheet. Excess label was removed by rinses and a 2-h wash in BSA solution. The dried sheets were detected by autoradiographic exposure of Dupont Cronex x-ray films using a Dupont Cronex Hi-Plus intensifying screen at -70°C.

RESULTS

Recruitment of Histone into Male Chromatin in the Absence of Protein Synthesis

We have shown the acquisition of cleavage stage histones by

the male pronucleus is independent of protein synthesis in polyspermi- cally fertilized eggs (29). To estimate a minimum amount of stored CS histones which could assemble into chromatin, we fertilized eggs to high degrees of polyspermy in the presence of emetine, a protein synthesis inhibitor. Radioactive amino acid incorporation was monitored to determine the extent of inhibition of protein synthesis under these conditions.

Two equivalent samples of eggs were incubated in NH₄Cl-seawater containing [¹⁴C]leucine. The cultures were polyspermi- cally fertilized to an average of 50 male pronuclei per egg, washed, and cultured for 60 min in seawater containing [¹⁴C]leucine. To one culture, 10⁻⁴ M emetine was added at the time of fertilization and was present in all subsequent solutions. The other culture was run in parallel without emetine to serve as a control. Incorporation of amino acid into protein was monitored at 15 min intervals. Crude nuclei were isolated from both cultures at 60 min postfertilization, and their histones were extracted and compared by two-dimensional gel electro- phoresis.

As seen in Fig. 1 *a* and *b*, the protein patterns of stained gels from these samples are virtually identical. As previously re- ported (29), sperm H1 has been replaced by CSH1, sperm H2B by proteins O and P, and protein N has accumulated on the chromatin in both samples. (O and P are probably modified SpH2B proteins and N is a stored protein that accumulates in the chromatin progressively during the first cell cycle.) The cleavage stage histone variants CSH2A and CSH2B are present at levels at least equal to the corresponding sperm histone variants (e.g., SpH₂A).

The amino acid incorporation curves (Fig. 2) for emetine- treated and control embryos from this culture indicate that emetine efficiently inhibited protein synthesis, even under conditions of high polyspermy. Thus, the chromatin remodeling of the male pronucleus in the first cell cycle is independent of protein synthesis and must, therefore, be accomplished by protein stored in the egg before fertilization.

The high degree of polyspermy in the experiment allows an

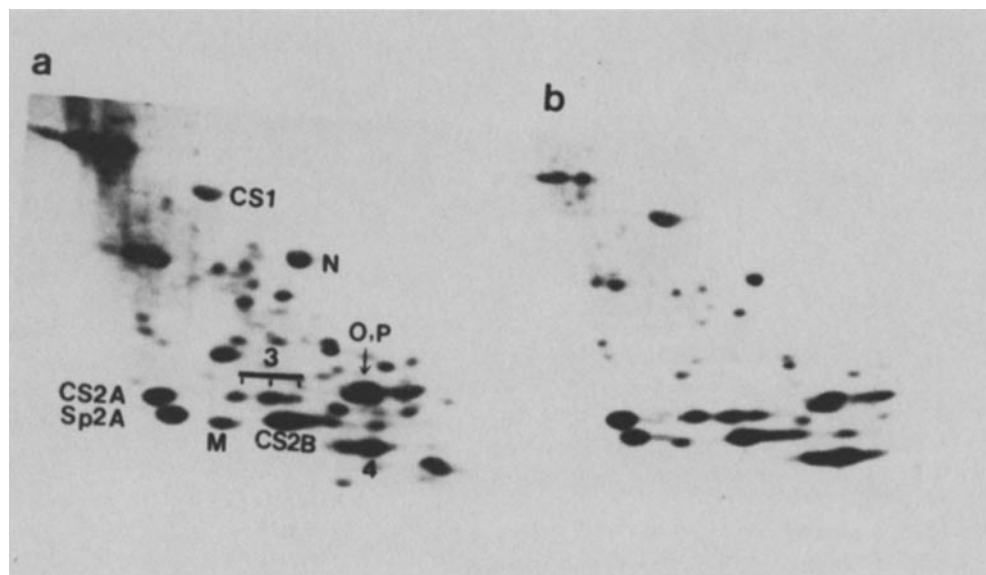


FIGURE 1 Male pronuclear histones in the absence of protein synthesis at high degrees of polyspermy. One culture was treated with 10⁻⁴ M emetine from the time of NH₄Cl activation until pronuclear isolation. An equivalent unexposed culture served as a control. The degree of polyspermy was ~50 pronuclei/egg. Pronuclei were isolated 60 min after fertilization. Two-dimensional gels of the protein were stained with Coomassie Blue. (a) Emetine nuclear proteins. (b) Control nuclear proteins.

estimate of the amount of stored functional histone present in the egg. Assuming a normal histone/DNA mass ratio in the emetine-blocked egg (29) and no replication, a degree of polyspermy of 50 means that at least 25 haploid amounts (~4–5

pg/egg/histone variant) of each incorporated CS histone species was stored in the egg. Because replication probably progressed under conditions of protein synthesis inhibition (45), 25 haploid equivalents would represent a minimum estimate.

Quantification of Stored Histone Acid-extracted from Whole Eggs

Proteins of electrophoretic mobilities corresponding to histone standards can be acid-extracted from whole eggs. When the proteins from this type of extraction are run on a two-dimensional polyacrylamide gel, a complex pattern is seen (Fig. 3 a). There are five proteins which co-migrate with known standards: CSH1, CSH2A, CSH2B, H3^{''}, and H4. (H3^{''} is the major H3 spot that accumulates during the first cell cycle.) The standards used for comparison are taken from two-cell embryo nuclei.

Fig. 3 b shows an autoradiograph of the nuclear proteins in two-cell embryos synthesized during this period. The CS and α histone spots from the gel co-migrate with protein standards taken from blastula chromatin. The α variants are at a higher specific activity as reported by Newrock et al. (19). Comparison of the autoradiograph with the total acid-extracted proteins shows that no protein spots co-migrating with the α -, β -, γ -, or δ -variants of H1, H2A, or H2B are found in the egg (see reference 29). In addition, proteins O, P, and N, prominent components of 60-min pronuclear chromatin (29), do not seem to be synthesized in the early embryo. Therefore, these proteins used in the early remodeling of the male pronuclear chromatin must come either from stored pools in the egg or from the modification of sperm protein.

To quantify the amount of protein in each putative histone spot, we extracted overnight in 0.4 N H₂SO₄ a known number of eggs. The acid-soluble proteins were precipitated, dried, and dissolved in the minimum amount of sample buffer to solubilize the entire pellet. Aliquots were run on two-dimensional gels.

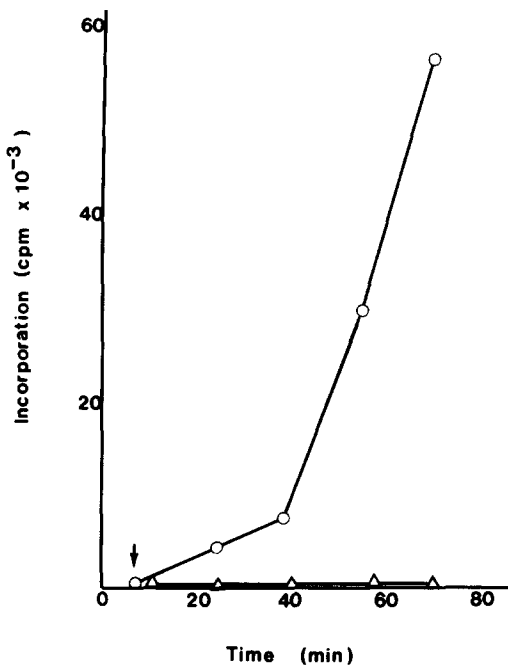


FIGURE 2 Inhibition of protein synthesis with 10^{-4} M emetine. Protein synthesis was measured as the incorporation of radioactive amino acid into TCA-precipitable material. [¹⁴C]leucine (0.5 μ Ci/ml) was added to control and emetine-treated cultures 10 min before fertilization. The embryos were then labeled continuously until pronuclear isolation. The arrow marks the time of fertilization. Abscissa: time after NH₄Cl activation (min). Ordinate: cpm incorporated $\times 10^{-3}$. Δ , emetine-treated embryos; \circ , control embryos.

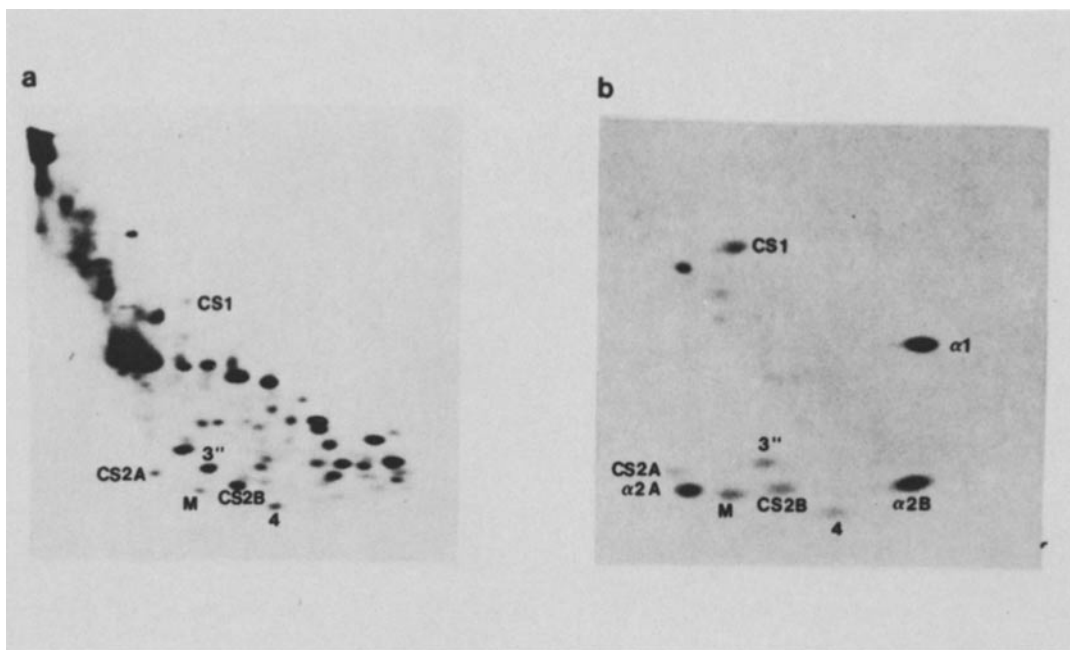


FIGURE 3 The comigration of spots from whole-egg acid extracts with CS histone from 2-cell embryo standards. (a) Two-dimensional gel pattern of proteins extracted from whole eggs with 0.4 N H₂SO₄. Spots that comigrate with the CS histones are indicated. (b) Autoradiograph of the two-dimensional gel pattern of histones labeled with [¹⁴C]arginine and [¹⁴C]lysine up to the 2-cell stage. The spots of high specific activity are the α histones; the spots of lower specific activity are the CS histones.

The amount of histone protein in each of the five histone spots was estimated by comparing the staining intensity of these spots and the staining intensity of known quantities of sperm histones (Fig. 4). The sperm histones were serially diluted and run in parallel with samples of the acid extract. Gel scans showed that the four sperm core histones were present in equal amounts.

In the egg extracts, spots corresponding to H3^{''} and CSH2B are roughly twice as intense as spots corresponding to H4 and

CSH2A. The unequal ratios might be real or might arise either from incomplete extractability of some of the histones or from contamination of the others by proteins of equivalent electrophoretic mobilities. Recovery of exogenously added trace amounts of ¹⁴C-blastula histones was 40–60%. Few proteins in the cells should be basic enough or of sufficiently low molecular weight to run in the histone region of the gel system used, which was in fact designed to eliminate contamination by ribosomal basic proteins (34).

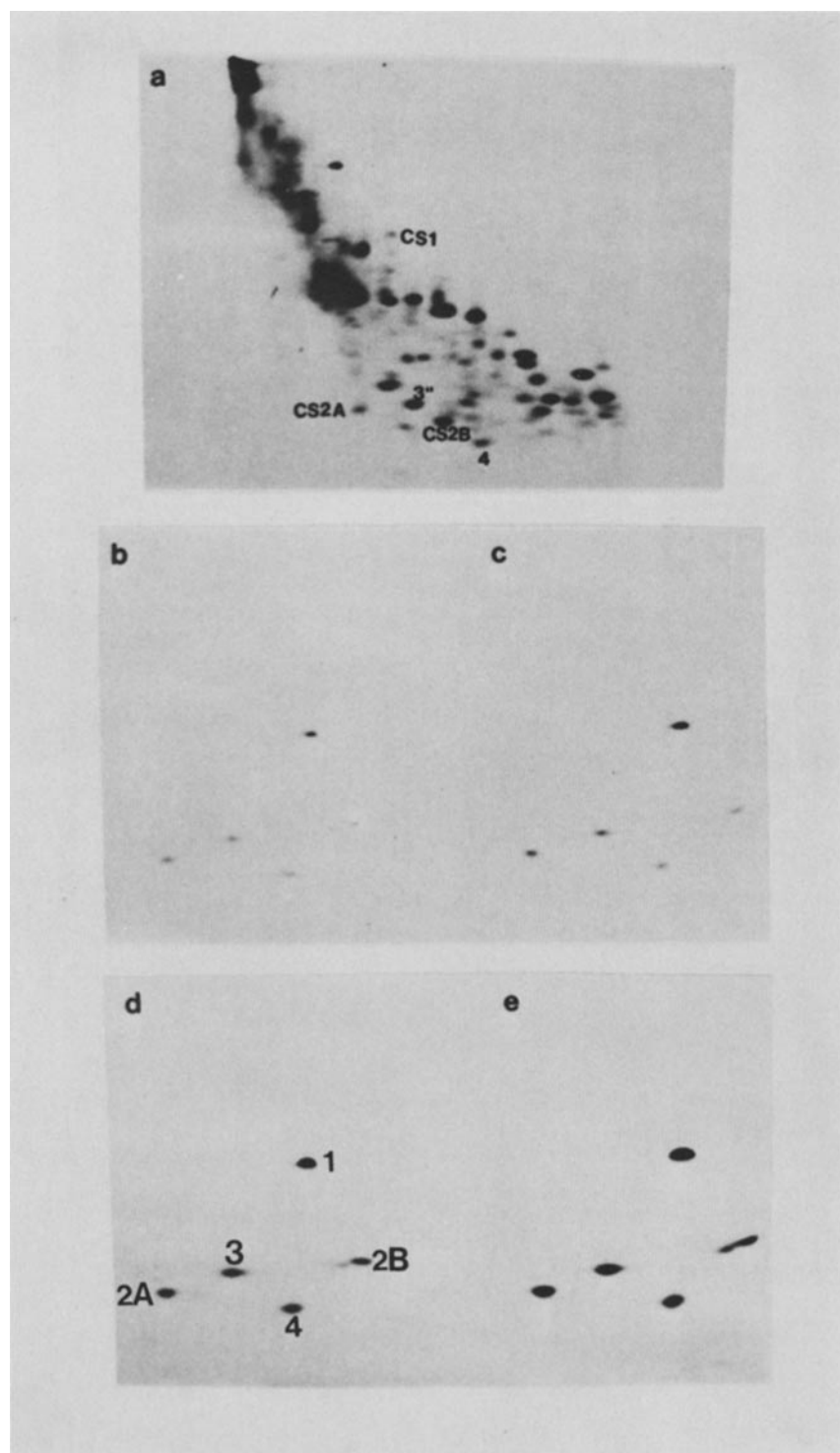


FIGURE 4 Quantitation of stored histone. (a) Two-dimensional gel of whole-egg acid extract. (b-d) Serial dilutions of a known concentration of sperm histones. (b) 1 μ g total sperm histone; (c) 2 μ g; (d) 4 μ g; and (e) 8 μ g.

To estimate the amount of stored histone per egg, we assume that each putative histone spot is uncontaminated and that recovery will be 50%. CSH2B in the egg extract, for example, corresponds in intensity to one spot in the 8 μg sperm histone standard ($\sim 8/5 \mu\text{g}/\text{spot}$). The CSH2B was extracted from the equivalent of 3.4×10^4 eggs, thus representing $(8/5 \mu\text{g} \times 2)/3.4 \times 10^4$ or $\sim 10^{-4} \mu\text{g}$ per egg corrected for recovery. Because the haploid amount of nuclear DNA in *S. purpuratus* is $0.77 \times 10^{-6} \mu\text{g}$ (53), a haploid equivalent of one of the five histones would be $\sim 0.15 \times 10^{-6} \mu\text{g}$. CSH2B is stored in amounts of $10^{-4}/0.15 \times 10^{-6}$ or 660 haploid equivalents as is H3". H4 and CSH2A would be present in half this amount. CSH1 is more difficult to estimate from staining intensity because its size is much larger than any corresponding sperm standard, but it too seems to be stored in relatively large amounts. Thus, enough histone may be stored to potentially fully complex with a few hundred diploid nuclei.

Immunochemical Identification

The immunochemical technique used to identify spots involves electrophoretic transfer from two-dimensional gels to nitrocellulose filters. The antigenic sites of bound proteins are accessible to antibody binding, which is detected by autoradiography after reaction with ^{125}I -protein A.

Transfer conditions were chosen such that sufficient protein would remain in the acrylamide gel for detection by staining with Coomassie Blue. This stained gel served as a template to align the autoradiograph. Fig. 5 shows a blot in which the filter was stained with Coomassie Blue to show that the protein two-dimensional pattern is transferred with minimal distortion. However, low molecular weight proteins transfer more rapidly onto the nitrocellulose filter so there is a slight enhancement of these spots. Therefore, the stained acrylamide gel from each transfer could be used as a replica that is superimposed over the autoradiogram for identification of the protein to which the antibody is bound.

Antihistone H4 was first tested on gel blots of histones isolated from sea urchin blastulae grown in the presence of actinomycin D. The actinomycin treatment causes an enrichment in CS histones. Fig. 6 C shows an overexposed autoradiogram and the stained replica gel of the actinomycin D sample (Fig. 6 B); obviously, the antibody is highly specific for H4

because no other histone species crossreacts even to a small extent. Fig. 7 is a set of serial dilutions of sperm histones. Both the gel and the corresponding autoradiogram are shown. Using these serial dilutions, we calculate the minimum sensitivity of the H4 antibody technique to be better than $2 \times 10^{-4} \mu\text{g}$ of protein.

Two-dimensional gels of sperm histones and acid-extractable proteins from whole eggs were transferred to nitrocellulose and reacted with H4 antibody. Fig. 8 a shows the stained gel pattern of the sperm histones with the accompanying autoradiograph (Fig. 8 b). Running the proteins on two-dimensional gels does not seem to alter the antibody recognition or specificity. Fig. 8 c and d show the stained acrylamide gel of total acid extracts of whole eggs after transfer with the accompanying autoradiograph of the nitrocellulose filter. By superimposing the two patterns, using the marked corners for proper orientation, it was found that the one reactive site on the filter is at the position of the lightly stained putative H4 seen on the gel. The antibody has maintained an extremely high degree of specificity when presented with the enormous number of background proteins of similar solubility and molecular weight.

These antibody data thus confirm the identity of the protein that we had tentatively labeled H4 in the pattern of acid-extractable proteins from whole eggs. This also supports the previous identification of histone proteins by mobility on two-dimensional gels.

Although the estimates of the amount of histones in whole eggs indicate that they are well over the amount expected for the haploid maternal chromosomes, it was not known whether any of this excess histone was cytoplasmically localized. To determine this, we broke eggs into nucleated and enucleated fragments by centrifugation in a sucrose gradient. The enucleated merogons were washed free of sucrose and extracted in acid as described for whole eggs.

The acid-extractable proteins run on two-dimensional gels had a protein pattern almost identical to the pattern of proteins from whole eggs, including the five spots that co-migrate with the stored histones (Fig. 9 a). The gel was transferred and reacted with H4 antibody (Fig. 9 b). Comparison of the stained gel and the autoradiograph indicates that the protein reacting with the antibody is the protein which co-migrates with histone H4.

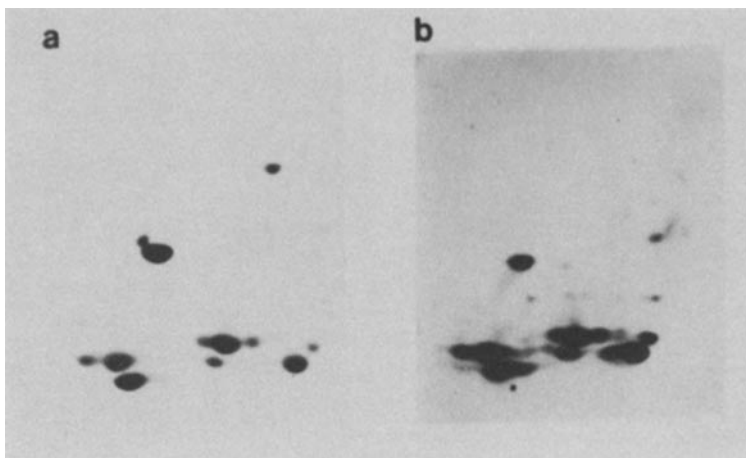


FIGURE 5 Electrophoretic transfer from a two-dimensional polyacrylamide gel to a nitrocellulose filter. Proteins extracted from actinomycin D-treated blastula nuclei were run on a two-dimensional polyacrylamide gel and then electrophoretically transferred to a nitrocellulose filter. Both the gel and the filter were stained after the completion of the transfer. (a) Coomassie Blue-stained gel. (b) Coomassie Blue-stained filter.

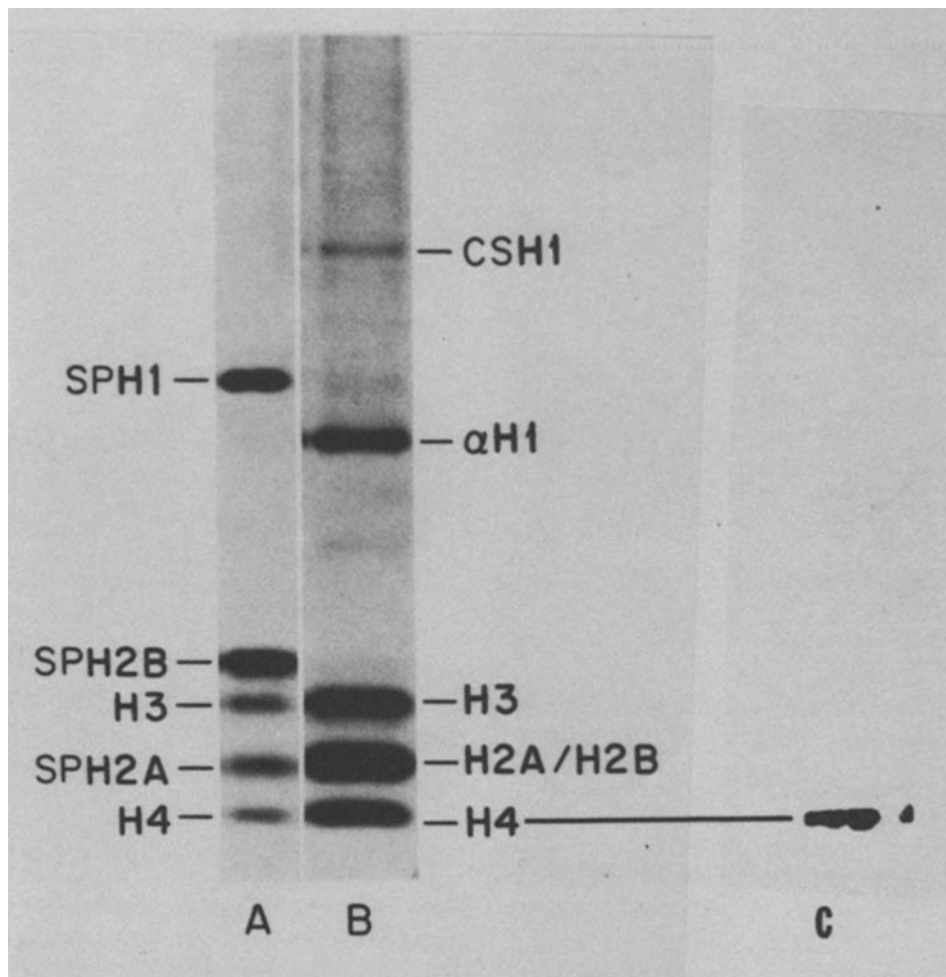


FIGURE 6 H4 antibody characterization. H4 antibody was incubated with the histones extracted from actinomycin D-treated blastula embryos. The histones were separated on a one-dimensional SDS gel and transferred to a nitrocellulose filter. The filter was reacted with ^{125}I -labeled *Staphylococcus aureus* A protein. (a) SDS gel of sperm histone standard stained with Coomassie Blue. (b) Stained SDS gel of actinomycin D histones. (c) Autoradiograph of H4 antibody reaction with actinomycin D blastula histones (replica of gel B). Note the specificity of the antibody for histone H4.

Therefore, at least some of the stored histone appears to be cytoplasmic. It is not possible to say how much of the stained protein is cytoplasmic, for two reasons. First, the egg initially breaks into roughly equal size fragments so that a substantial portion of the cytoplasm is included in the nucleated merogon. Secondly, it is difficult to know the number of eggs from which the enucleated halves are derived because some of them break a second time into quarters.

H2B Antibody Binding

A second antibody was utilized to confirm the identity of the histones in total acid extracts from eggs. The antibody used was raised against calf thymus H2B. Preliminary experiments indicated that against calf thymus histones the antibody preparation had a low affinity for all the histones but very high affinity for calf thymus H2B. When reacted with sea urchin sperm standard, however, the antibody did not react strongly with any of the histones. The cross-reactivity with nonhomologous histones is presumably because the histones share common sequences which the antibody may recognize. Therefore, the antibody behaves as a reagent of broad specificity for histones.

The antibody was reacted with the proteins from whole eggs. The stained gel and the resulting autoradiographic pattern are shown in Fig. 10. The autoradiograph revealed three proteins most reactive with the antibody. Superimposition of the reference gel pattern revealed that two of these were the proteins tentatively designated CSH2B and H3". The third reactive protein runs in the region in which we find pronuclear proteins O and P, which take the place of sperm H2B following fertilization (29).

The fact that only three proteins in the histone region among the hundreds of background proteins were most reactive with this antibody indicates a very specific reaction. Their antigenicity with a histone antibody further supports the identification of the three spots as histones.

DISCUSSION

We have demonstrated by two different procedures that histone proteins are stored in unfertilized sea urchin eggs in considerable excess over that required for a haploid amount of nuclear DNA. Based upon solubility in 0.4 N H_2SO_4 , two-dimensional electrophoretic co-migration with in vivo labeled embryo histone standards, and antigenicity, we have identified storage of

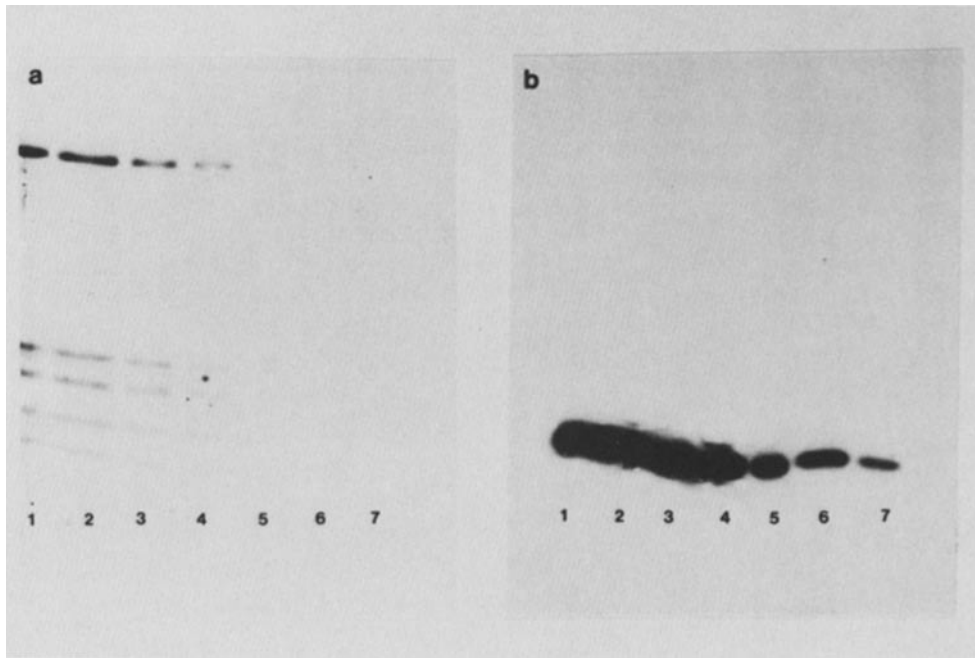


FIGURE 7.

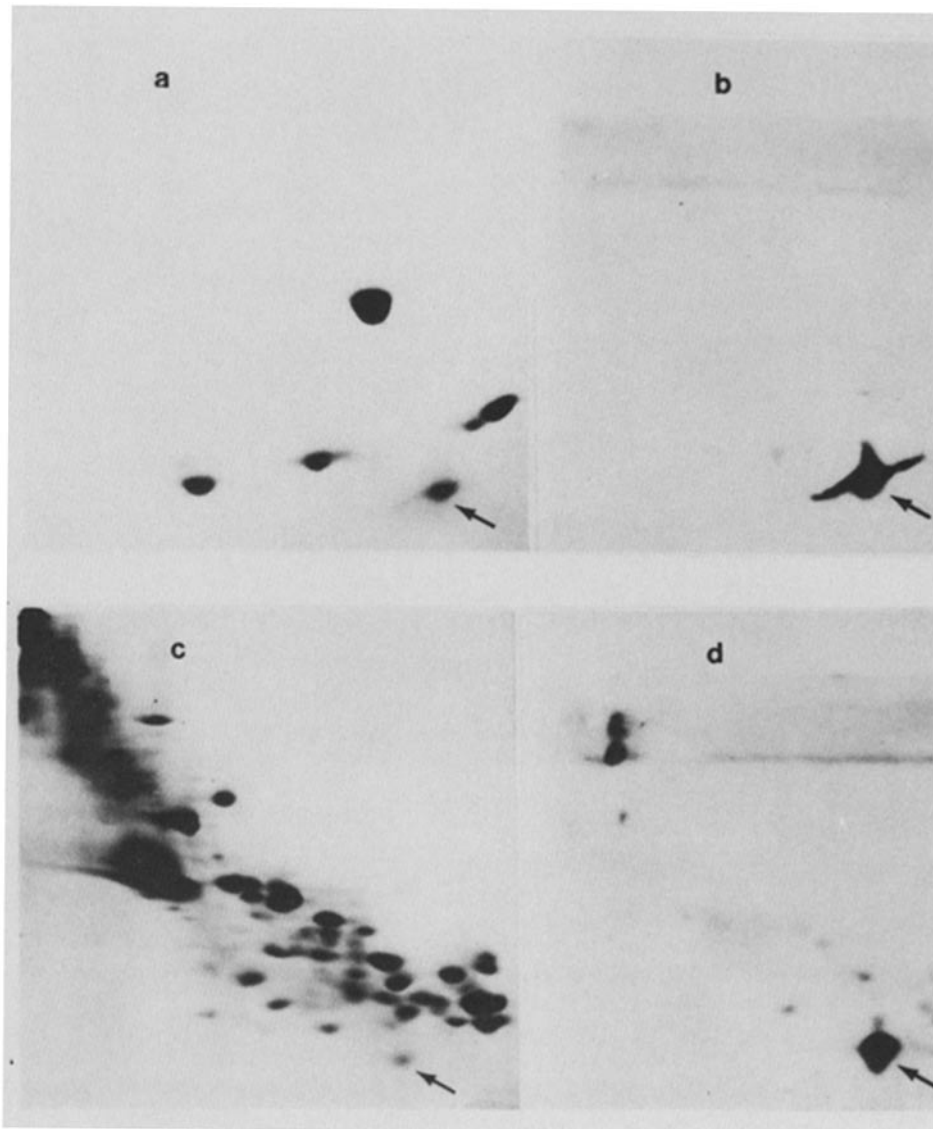


FIGURE 8.

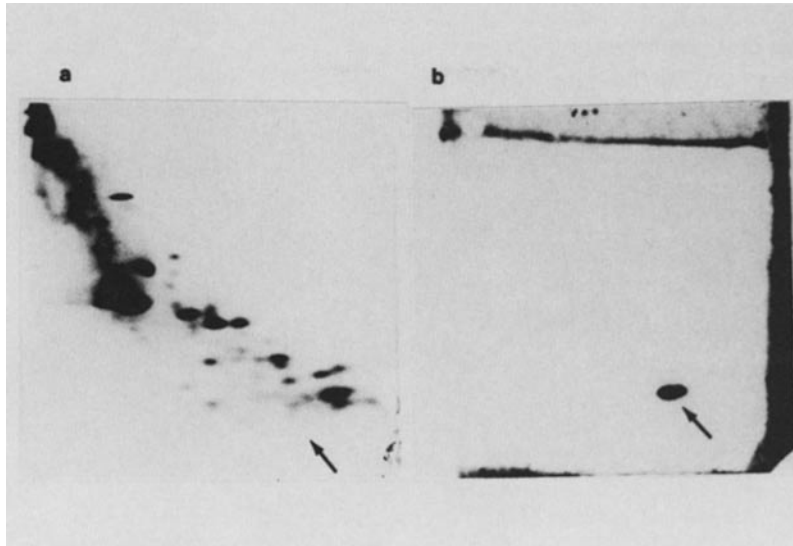


FIGURE 9 Antibody identification of H4 histone in the acid extract of enucleated egg halves. (a) Coomassie Blue-stained gel of the acid extracts from enucleated egg fragments after transfer; the arrow points to the protein spot which corresponds to the spot on the autoradiogram. (b) Autoradiogram. The antibody technique allows detection of a protein spot which is barely visible with Coomassie Blue staining.

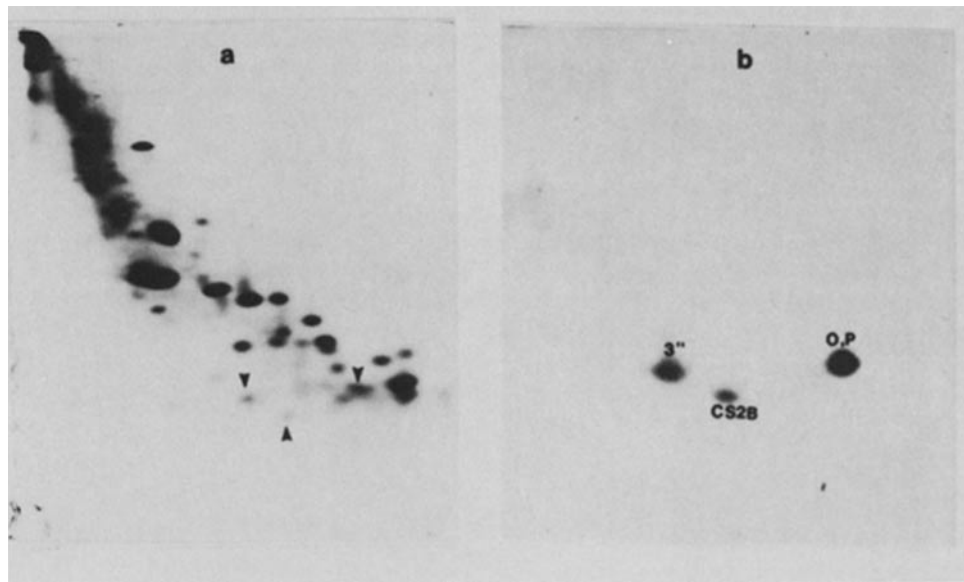


FIGURE 10 H2B antibody reaction with protein of acid extracts from the whole eggs. (a) Coomassie Blue-stained gel after transfer. (b) Autoradiogram of the corresponding nitrocellulose filter after reacting with antibody. One spot corresponds to the H3'' and another comigrates with the CSH2B. The third spot corresponds to the region of the gel where pronuclear proteins O,P run.

FIGURE 7 Characterization and sensitivity of H4 antibody. Sperm histones were serially diluted and electrophoresed on a one-dimensional SDS gel. These proteins were transferred to a nitrocellulose filter which was reacted with H4 antibody. (a) Coomassie Blue-stained acrylamide gel. (1) 4 μ g sperm histone; (2) 2 μ g; (3) 1 μ g; (4) 0.05 μ g; (5) 0.02 μ g; (6) 0.01 μ g; (7) 0.001 μ g. (b) Autoradiogram of the corresponding lanes of the nitrocellulose filter.

FIGURE 8 Identification of H4 histone in total acid extracts from whole eggs using the H4 antibody. Sperm histones and acid-extractable proteins from eggs were separated on two-dimensional gels, electrophoretically transferred to nitrocellulose filters, and reacted with H4 antibody. (a) Coomassie Blue-stained gel of sperm histone after transfer. The arrow indicates the protein spot recognized by the antibody. (b) Autoradiogram of the corresponding nitrocellulose filter. (c) Coomassie Blue-stained gel of egg acid-extract after transfer. (d) Autoradiogram of the corresponding nitrocellulose filters.

CSH1, CSH2A, CSH2B, H3, and H4. Quantification by comparison to sperm histone standards indicates that as much as 300–600 haploid equivalents of some of these histones may be stored.

We have also shown that at least a fraction of this stored histone is capable of associating with male pronuclear DNA so as to copurify with nuclei and chromatin (29). A minimum estimate of the assembly competent store was made from chromatin isolated from cells blocked in protein synthesis. Sufficient stored forms of histone were found in the chromatin of a single polyspermic egg to supply at least 25 haploid nuclei. This estimate is conservative, for two reasons. It assumes that the male pronuclei have not replicated and that the storage forms have replaced half of the sperm histones of the 50 male pronuclei. Although we have no measure of sperm histone turnover or the degree of replication which might have occurred, it is known that in monospermically fertilized eggs blocked in protein synthesis DNA synthesis appears essentially unaffected (45). Assuming no turnover of, for example, sperm H2A, the amount of CSH2A incorporated into the chromatin under protein synthesis blockade could be as much as 50 haploid equivalents. This amount of maternal histone might easily be accommodated in the male pronuclei if a large fraction of the 50 haploid DNA were replicated. In addition, a higher histone-to-DNA ratio may be required in the pronucleus because of the smaller nucleosome repeat distance in the pronuclear chromatin compared to that of sperm (46). Most importantly, 25 haploid equivalents represent an underestimate, because the polyspermic eggs have probably not been saturated with the upper limit of male pronuclei which they might accommodate.

A pool of stored histones has been demonstrated in *Xenopus* oocytes (28). This is in direct contrast to most other eukaryotic cells studied in which histone synthesis and DNA synthesis are tightly coupled processes, with little if any storage of the histones (25, 47). Thus, replication in the absence of protein synthesis results in histone-depleted DNA which is more susceptible to digestion with Staphylococcal nuclease (48, 49). However, neither sea urchin embryos (26, 27) nor *Xenopus* embryos (50) display the tight coupling of histone and DNA synthesis characteristic of somatic cells.

There may be several reasons for the anomalous situation found in oocytes. The most obvious is that stored histones as well as stored histone message are needed to support the rapid replication cycles which begin following fertilization. In *Xenopus* it has been estimated that early replication cycles could not be supported solely by the synthesis of new histone protein (50).

However, the upper estimate of, for example, stored CSH2B is ~350 diploid DNA equivalents. This would be more than enough CS histone to fully complex with the DNA utilized in development to the 250-cell blastula stage. However, blastula chromatin is comprised of predominantly α histone variants; cleavage stage histones contribute only a small fraction of the total histone complement (19). A second possibility is that the stored histone is needed to accomplish the early histone transitions on the male pronucleus, transitions which may be essential for the reactivation and chromatin structural modeling of this pronucleus (46). It is a specialized set of variants (CS) which is both stored and participating in the first stages of remodeling. The large amounts of stored cleavage stage histones may be necessary to achieve high enough concentrations in the large egg cell to permit exchanges such as the CSH1

replacement of sperm H1, or to insure proper placement of the core proteins during replication, when nucleosome spacing becomes significantly shortened (46).

The fate of the excess stored histones has not been investigated. Once incorporated into the chromatin, embryonic histones seem to remain there for many cell generations (16, 17). However, it is possible that a slow influx of the stored histones over the entire period of larval development (>20,000 cells) might result in the utilization of the entire stored pool (14, 51).

One interesting finding resulting from the H2B antibody reaction was the identification of stored protein in the region of the gel where pronuclear proteins O and P run. These proteins were previously suggested to be modified forms of the sperm H2B proteins (29) and are shown by this work not to be synthesized during the first few cell cycles following fertilization (Fig. 3b). If, in fact, these represent H2B variants stored in the egg and not modifications of sperm H2B, their replacement of sperm H2B histones in male pronuclear chromatin protein during the G₁ phase of the first cell cycle would be a unique case of core histone protein exchange. Such an exchange does not occur even in heterokaryons after rounds of replication (52). Clearly, further protein chemistry including peptide mapping must be done to verify the identity of O and P as a separate, stored histone variant class.

L. Herlands would like to thank V. G. Allfrey for his support and Rosemarie J. Gruss for her help in the computer scanning of gels.

This work was supported by National Institutes of Health grant HD 12982 to D. Poccia.

Received for publication 28 January 1981.

REFERENCES

- Hinegardner, R. T., B. Rao, and D. E. Feldman. 1964. The DNA synthetic period during early development of the sea urchin egg. *Exp. Cell Res.* 36:53–61.
- Loeb, L. A., D. Mazia, and A. D. Ruby. 1967. Priming of DNA polymerase in nuclei of sea urchin embryos by native DNA. *Proc. Natl. Acad. Sci. U. S. A.* 57:84–88.
- Loeb, L. A., B. Fansler, R. Williams, and D. Mazia. 1969. Sea urchin nuclear DNA polymerase I. Localization in nuclei during rapid DNA synthesis. *Exp. Cell Res.* 57:298–304.
- Fansler, B. A., and L. A. Loeb. 1969. Sea urchin nuclear DNA polymerase. II. Changing localization during early development. *Exp. Cell Res.* 57:305–310.
- Nemer, M. 1962. Characteristics of the utilization of nucleosides by embryos of *Paracentrotus lividus*. *J. Biol. Chem.* 237:143–149.
- Kavanau, J. L. 1956. Metabolic patterns in the sea urchin embryos. *Develop. Biol. Conf. Ser. Univ. of Chicago Press, Chicago, Illinois.*
- Brachet, J. 1967. Effects of hydroxyurea on development and regeneration. *Nature (Lond.)* 214:1132–1133.
- Gross, P. R., and G. H. Cousineau. 1963. Effects of actinomycin D on macromolecule synthesis and early development in sea urchin eggs. *Biochem. Biophys. Res. Commun.* 10:321–326.
- Gross, P. R., and G. H. Cousineau. 1964. Macromolecule synthesis and the influence of actinomycin on early development. *Exp. Cell Res.* 33:368–395.
- Skoultchi, A., and P. R. Gross. 1973. Maternal histone messenger RNA: detection by molecular hybridization. *Proc. Natl. Acad. Sci. U. S. A.* 70:2840–2844.
- Gross, K., J. Ruderman, J. Jacobs-Lorena, C. Baglioni, and P. R. Gross. 1973. *Nature (Lond.)* 241:272–274.
- Lifton, R. P., and L. H. Kedes. 1976. Size and sequence homology of masked maternal and embryonic histone messenger RNAs. *Dev. Biol.* 38:47–55.
- Arceci, R. J., and P. R. Gross. 1980. Histone variants and chromatin structure during sea urchin development. *Dev. Biol.* 80:186–209.
- Kedes, L. H., P. R. Gross, G. Cognetti, and A. L. Hunter. 1969. Synthesis of nuclear and chromosomal proteins on light polyribosomes during cleavage in the sea urchin embryo. *J. Mol. Biol.* 45:337–351.
- Hill, R. J., D. L. Poccia, and P. Doty. 1971. Towards a total macromolecular analysis of sea urchin embryo chromatin. *J. Mol. Biol.* 61:445–462.
- Ruderman, J. V., and P. R. Gross. 1974. Histones and histone synthesis in sea urchin development. *Dev. Biol.* 36:286–298.
- Poccia, D. L., and R. T. Hinegardner. 1975. Developmental changes in chromatin proteins of the sea urchin from blastula to mature larva. *Dev. Biol.* 45:81–89.
- Cohen, L. H., K. M. Newrock, and A. Zweidler. 1975. Stage specific switches in histone synthesis during embryogenesis of the sea urchin. *Science (Wash. D. C.)* 190:984–997.
- Newrock, K. M., C. R. Alfageme, R. V. Nardi, and L. H. Cohen. 1978. Histone changes during chromatin remodelling in embryogenesis. *Cold Spring Harbor Symp. Quant. Biol.* 42:421–431.
- Newrock, K. M., L. H. Cohen, M. B. Hendricks, R. J. Donnelly, and E. S. Weinburg. 1978. Stage specific mRNAs coding for subtypes of H2A and H2B histones in the sea urchin embryo. *Cell* 14:327–336.
- Childs, G., R. Maxson, and L. H. Kedes. 1979. Histone gene expression during sea urchin

- embryogenesis: isolation and characterization of early and late messenger RNAs of *Strongylocentrotus purpuratus* by gene specific hybridization and template activity. *Dev. Biol.* 73:153-173.
22. Hieter, P. A., M. B. Henricks, K. Hemminki, and E. S. Weinberg. 1979. Histone gene switch in the sea urchin embryo. Identification of late embryonic histone messenger ribonucleic acids and the control of their synthesis. *Biochemistry*. 18:2707-2716.
 23. Arceci, R. J., D. R. Senger, and P. R. Gross. 1976. The programmed switch in lysine-rich histone synthesis at gastrulation. *Cell*. 9:171-178.
 24. Kunkel, N. S., and L. S. Weinberg. 1978. Histone gene transcription in the cleavage and mesenchyme blastula embryo of the sea urchin *S. purpuratus*. *Cell*. 14:313-326.
 25. Robbins, E., and T. Borun. 1967. The cytoplasmic synthesis of histones in HeLa cells and its temporal relationship to DNA replication. *Proc. Natl. Acad. Sci. U. S. A.* 57:409-412.
 26. Nishioka, D., and D. Mazia. 1977. The phosphorylation of thymidine and the synthesis of histones in ammonia-treated eggs and egg fragments of the sea urchin. *Cell Biology International Report*. 1:23-30.
 27. Arceci, R. J., and P. R. Gross. 1977. Noncoincidence of histone and DNA synthesis in cleavage cycles of early development. *Proc. Natl. Acad. Sci. U. S. A.* 74:5016-5020.
 28. Adamson, E. O., and H. R. Woodland. 1977. Changes in the rate of histone synthesis during oocyte maturation and very early development of *Xenopus laevis*. *Dev. Biol.* 57:136-149.
 29. Poccia, D., J. Salik, and G. Krystal. 1981. Transitions in histone variants of the male pronucleus following fertilization and evidence for a maternal store of cleavage stage histones in the sea urchin egg. *Dev. Biol.* 82:287-296.
 30. Poccia, D., D. LeVine, and J. Wang. 1978. Activity of a DNA topoisomerase (nicking-closing enzyme) during sea urchin development and the cell cycle. *Dev. Biol.* 64:273-283.
 31. Mazia, D., J. M. Mitchison, H. Medina, and P. Harris. 1961. The direct isolation of the mitotic apparatus. *J. Biophys. Biochem. Cytol.* 10:467-474.
 32. Hogan, B., and P. R. Gross. 1971. The effect of protein synthesis inhibition on the entry of mRNA into the cytoplasm of sea urchin embryos. *J. Cell Biol.* 49:692-701.
 33. Fry, B. J., and P. R. Gross. 1970. Patterns and rates of protein synthesis in sea urchin embryos. II. Calculation of absolute rates. *Dev. Biol.* 21:125-146.
 34. Savić, A., and D. Poccia. 1978. Separation of histones from contaminating ribosomal proteins by two-dimensional gel electrophoresis. *Anal. Biochem.* 88:573-578.
 35. Bonner, C. M., and R. A. Laskey. 1974. A film detection method for tritium labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
 36. Lutin, W. A., C. F. Kyle, and J. A. Freeman. Quantitation of brain proteins by computer-analyzed two-dimensional electrophoresis. In *Developments of Biochemistry*. N. Catsim-poolas, editor. Elsevier North Holland, Inc., Amsterdam. 2:93-106.
 37. Krystal, G., and D. Poccia. 1979. Mitotic and premature chromosome condensation in the sea urchin egg. *Exp. Cell Res.* 123:207-219.
 38. Westerhuyzen, D. R., E. L. van der Bohm, and C. von Holt. 1974. Fractionation of chicken erythrocyte whole histone into the six main components by gel exclusion chromatography. *Biochim. Biophys. Acta.* 359:341-345.
 39. Stollar, B. D., and M. Ward. 1970. Rabbit antibodies to histone fractions as specific reagents for preparative and comparative studies. *J. Biol. Chem.* 245:1261-1266.
 40. Inami, Y. H., and R. M. Nakamura. 1976. Hemagglutination tests for detection of antibodies of deoxyribonucleic acid and nucleoprotein. In *Manual of Clinical Immunology* N. R. Rose and H. Friedman, editors. American Society of Microbiology.
 41. Rosenthal, J. D., K. Hayashi, and A. L. Noskins. 1972. Rapid micro-radioimmunoassay for the measurement of antiviral antibody. *J. Immunol.* 109:171-173.
 42. Romani, M., G. Vidali, C. S. M. Tahourdin, and M. Bustin. 1980. Solid phase radioimmunoassay for chromosomal components. *J. Biol. Chem.* 255:468-474.
 43. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76:4350-4354.
 44. Denhardt, D. 1966. A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-652.
 45. Waganar, E. B., and D. Mazia. 1978. The effect of emetine on first cleavage division in the sea urchin. In *ICN-UCLA Symposium on Molecular and Cell Biology XII: Cell Reproduction*. E. R. Dirksen, D. M. Prescott, and C. F. Fox, editors. Academic Press, Inc., N. Y. 539-546.
 46. Savić, A., P. Richman, P. Williamson, and D. Poccia. 1981. Alterations in chromatin structure during early sea urchin embryogenesis. *Proc. Natl. Acad. Sci. U. S. A.* In press.
 47. Kedes, L. 1976. Histone messages and histone genes. *Cell*. 8:321-331.
 48. Seale, R. L. 1976. Studies on the mode of segregation of histone nu bodies during replication in HeLa cells. *Cell*. 9:423-429.
 49. Weintraub, H. 1976. Cooperative alignment of nu bodies during chromosome replication in the presence of cycloheximide. *Cell*. 9:419-422.
 50. Adamson, E. O., and H. R. Woodland. 1974. Histone synthesis in early amphibian development. Histone and DNA synthesis are not coordinated. *J. Mol. Biol.* 88:263-285.
 51. Arceci, R. J., and P. R. Gross. 1980. Histone variants and chromatin structure during sea urchin development. *Dev. Biol.* 80:186-209.
 52. Manser, T., T. Thacher, and M. Rechsteiner. 1980. Arginine rich histones do not exchange between human and mouse chromosomes in haploid cells. *Cell*. 19:993-1004.
 53. Hinegardner, R. T. 1974. Cellular DNA content of the echinodermata. *Comp. Biochem. Physiol. B. Comp. Biochem.* 49:219-231.