Shift in Nucleosome Populations during Embryogenesis: Microheterogeneity in Nucleosomes during Development of the Sea Urchin Embryo†

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ABSTRACT: The chromatin subunit or nucleosome structure in the fertilized embryo of the sea urchin *Strongylocentrotus purpuratus* has been investigated during early stages of growth when the translation of mRNA for histone variants of H1, H2A, and H2B is markedly changing. It is shown that *nucleosomes exist prior to differentiation, in cells which are "totipotent"*. We have found that micrococcal nuclease derived monomer and multimeric nucleosomes can also be isolated from each stage of development, beginning with the two-cell embryo. Basic proteins in the nucleosome fractions have been analyzed directly by employing a new technique developed in our laboratory: protamine release of chromosomal proteins into Triton–acid–urea mini gels [Shaw, B. R., & Richards, R. G. (1979) *NATO Adv. Study Inst. Ser., Ser. A 21a*, 125–136]. These studies indicate that cleavage-stage proteins are nucleosomal proteins. Likewise, the α-type histone H2A variant and each of the putative late stage β, γ, and δ H2A proteins are major components of nucleosomes at the appropriate stages. It is also shown that the nucleosomal population is composed of those histone components that were synthesized up to and during that stage and that each type of histone variant which was synthesized at an earlier stage is *retained in the nucleosomal population* of a subsequent developmental stage. The nucleosome population at morula, blastula, and mesenchyme blastula also contains the cleavage-stage histones synthesized only during early development. Thus, even after their synthesis has ceased, cleavage-stage histone proteins remain associated with DNA in a nucleosomal type structure. These studies provide evidence for a *shift in the nucleosomal population* of a cell during embryogenesis and lend support to a functional role for the well-characterized switches in histone mRNA and translated histone protein products observed during embryogenesis in sea urchin nuclei.

Little is known as to how variations in histone subtypes other than H1 affect the structure of chromatin. Multiple forms of histones H2A, H2B, and H3, which differ by one or several amino acids in their primary sequence, have been identified in a number of organisms (Marzluff et al., 1972; Cohen et al., 1975; Blankstein & Levy, 1976; Blankstein et al., 1977; Franklin & Zweidler, 1977). The existence of multiple forms of histones implies the existence of multiple types of nucleosomes. To date, however, no isolation or characterization of diverse nucleosomal populations has been reported for any eukaryotic organism which exhibits changes in core histone subtypes. There are also no studies on the distribution of different core histone subtypes to nucleosomes and their effect on the nucleosomal structures. There is a lack of information as to (i) whether all the histonelike variants are localized to nucleosomes, (ii) whether they are segregated to different parts of chromatin, and (iii) whether the type of histone variant present will locally determine a unique structure for that chromatin. Exactly how the changes in histone variants might alter the structure of chromatin and influence their cellular function remains unknown. Since the appearance of new histone variants in sea urchin embryos correlates with specific developmental stages (Johnson et al., 1973; Seale & Aronson, 1973; Rudderman & Gross, 1974; Cohen et al., 1975; Newrock et al., 1978a), the possibility exists that histone variants might be involved in gene modulation during development. Zweidler (1980) has suggested that changes in the growth rate and development of cells are correlated with switches in histone variants.

It has previously been established that at the four-cell and mesenchyme blastula stage in sea urchin development there are shifts in the types of H1, H2A, and H2B histones synthesized, while histones H3 and H4 appear to remain unchanged (Cohen et al., 1975; Newrock et al., 1978a,b). Additionally, the types of mRNA's coding for the cleavage and α H1, H2A, and H2B histones are replaced by mRNA's coding for the β, γ, δ, and ϵ histones (Weinberg et al., 1978; Childs et al., 1979; Kedes, 1979; Hieter et al., 1979). These shifts [from cleavage stage (CS) to α and from α to β, γ, δ, and ϵ histones] have been postulated to result in patterning of chromatin for later development of the embryo. Thus, there was good reason to believe that (i) a new set of nucleosomes would be organized during the shift at mesenchyme blastula stage from α to β, γ, and δ, and (ii) the old (CS and α) histones from cleavage stage through blastulation would remain in a nucleosomal structure at later times. We present evidence in support of both these predictions.

Other investigators have proposed a nucleosomal subunit structure for sea urchin at the morula and subsequent developmental stages based on the size of the DNA fragments released by micrococcal nuclease (Spadafora et al., 1976, 1978; Keichline & Wassarman, 1977, 1979). The assumption has been made that all of the core histone variants are in a nucleosomal arrangement. However, since only the DNA was examined and nucleohistone fragments were not isolated, and since over 50% of the chromatin DNA became resistant to micrococcal nuclease as development proceeded, the possibility existed that only one major type of histone variant was giving rise to the observed nucleosomal pattern, with the other types of histonelike proteins associated with DNA but in a non-nucleosome-type structure. In addition, no studies have been done to investigate the structure of chromatin in premorula embryos where CS proteins have been shown to be present. This stage of development is particularly important, for the

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question arises as to what the chromatin or nucleosome structure of the early embryo will be, when at fertilization the DNA (and, presumably, the histones also) is derived from two distinctly different cell types (sperm and egg), both having a chromatin structure different from that of the hatched blastula embryo as indicated by comparing nuclease accessibility and DNA repeat lengths (Spadafora & Geraci, 1975; Spadafora et al., 1976; Albanese et al., 1980; Honda et al., 1974; Cornudella & Rocha, 1979).

We therefore undertook a study of the nucleosome composition of sea urchin embryos during embryogenesis. As an initial step in addressing the question of nucleosomal heterogeneity, we have investigated the chromatin structure and the distribution of histone variants to nucleosomes during the early development of sea urchin embryos and report our results here. We have been especially interested in studying the role of the histonelike proteins (CS proteins) synthesized in the first few cleavages after fertilization. We have also asked whether the CS proteins are indeed components of nucleosomes (and, therefore, "histones") and whether these cleavage-stage proteins are retained in the nucleosomes at later stages.

Materials and Methods

Isolation of Nuclei and Chromatin. *Strongylocentrotus purpuratus* sea urchins were obtained from the Pacific Bio-Marine Laboratory, Venice, CA. Eggs were harvested by gently shaking the isolated gonads in artificial sea water and filtering through a fine nylon mesh. They were fertilized (to 95%) and grown to the appropriate stage at 5000 embryos/mL in Instant Ocean sea water at 16 °C. Embryos were labeled at the appropriate stage of development with either 1 mCi/L [^{3}H]thymidine (54 Ci/mmol) (New England Nuclear) or 0.5 mCi/L [^{3}H]leucine (58.5 Ci/mmol) and chased when necessary with two rinses of sea water containing 10 mM leucine and resuspended in the same medium. Embryos that were harvested prior to blastulation were treated at fertilization with 3 mM *p*-aminobenzoic acid–0.01 M Tris, pH 8.0, in sea water or 1 mM aminotriazole (Showman & Foerder, 1979) or grown in "hatching enzyme" to facilitate removal of the fertilization envelope.

The nuclear isolation method employing LiCl and low pH (Albanese et al., 1980) was used since it is effective in reducing endogenous nuclease activity during isolation of sea urchin nuclei. All operations were carried out at 0–5 °C. Embryos at the appropriate stage were harvested by low-speed centrifugation, and the pellet was resuspended with a Pasteur pipet and washed in 20 volumes of IG buffer [0.1 M sodium phosphate, 0.1 M LiCl, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF), pH 6.0] supplemented with 0.024 M EDTA, pH 6.0; the embryos were pelleted at 5000 rpm (4000*g*) in a Sorvall HB-4 rotor for 10 min. This step was repeated. The embryos were washed 1 time in IG buffer without EDTA, centrifuged as before, and homogenized in 20–40 volumes of IG buffer using a Dounce homogenizer with the tighter pestle. The number of strokes was determined by monitoring the extent of lysis through the microscope and ranged from 2 in the earlier stages to 8–10 for later stage (gastrula) embryos. Triton X-100 was then added (to a final concentration of 0.2%) to the broken cell suspension, and the nuclei were allowed to incubate for a further 5 min at 0 °C, after which the nuclei were pelleted at 4000 rpm (2600*g*) for 10 min. At this point, the nuclei were carefully resuspended with a Pasteur pipet in IG buffer in order to remove the Triton and centrifuged as before. This step can be repeated once more. Occasionally, the nuclei were sedimented through a 5-mL cushion of 0.5 M sucrose in IG buffer.

Nuclease Digestion of Nuclei. For all experiments, the nuclei were washed twice in KPS buffer [0.085 M KCl, 5 mM 1,4-piperazinediethanesulfonic acid·hydrochloride (Pipes·HCl), pH 7.0, and 0.3 M sucrose] and sedimented at 5000 rpm. They were resuspended in KPS buffer plus 1 mM $CaCl_2$ to a concentration of 1–3 mg of DNA/mL and digested with micrococcal nuclease (Worthington) at 37 °C for varying extents of time. Aliquots were removed at different time points, and the digestion was terminated by adding 0.1 volume of 30 mM EDTA. For DNA sizing, the nuclear digestion mixture was treated with 10 μg/mL pancreatic ribonuclease A at 37 °C for 30 min. Sodium dodecyl sulfate ($NaDodSO_4$) was added to a final concentration of 1%, and after 0.5 h, proteinase K was added to 100 μg/mL. The mixture was incubated at least 3 h at 37 °C and extracted once with phenol and 3 times with isoamyl alcohol:chloroform, 1:24. DNA pellets obtained after ethanol precipitation at −20 °C were washed once with 95% ethanol and dried.

Gel Electrophoresis of DNA Fragments. Three or four percent polyacrylamide [acrylamide:bis(acrylamide) 19:1] slab gels (13 × 13 × 0.15 cm^3) in E buffer (1 mM EDTA, 20 mM sodium acetate, and 40 mM Tris, pH 7.2) (Loening, 1967) were used for electrophoretic sizing of DNA fragments according to Shaw et al. (1976a). The gels were preelectrophoresed for 1 h at 80 V, and samples were electrophoresed for 3 h at 100 V. Samples of 3–25 μg of purified sea urchin DNA (dissolved in 30 μL of 0.1 E buffer plus 8% glycerol and 0.02% bromphenol blue) were applied per slot. The gels were stained with ethidium bromide (1 μg/mL for 30 min) following electrophoresis and photographed with a red Wratten filter on a low-wavelength UV light box (Model C-62 or C-63, UV Products). The sizes of the DNA fragments were calculated by comparing the mobilities of the fragments with those of restriction fragments of PM2-*Hae*III of known length (Noll, 1976). Autofluorography of ^{3}H-labeled DNA was carried out with preflashed Kodak Royal RP-XOMat film according to Bonner & Laskey (1974).

Fractionation of Nucleosomes on Sucrose Gradients. The digested nuclei or chromatin mixture was dialyzed overnight against 0.2 mM EDTA–2 mM Tris-HCl, pH 7.5, and pelleted at 10000*g* for 10 min. Nucleosomes in the supernatant were isolated on 10 mM Tris–0.7 mM EDTA, pH 7.2, sucrose gradients isopycnic for particle density 1.51 (Noll, 1974) and run at 26 500 rpm for 18 h in an SW 27 rotor [following the method of McCarty et al. (1974)].

Electrophoresis of Nucleoproteins. Nucleoprotein electrophoresis was carried out at 25 °C using 4.0% acrylamide [acrylamide:*N*,*N*′-methylenebis(acrylamide) 25:1] tube gels (12 × 0.3 cm i.d.) or slab gels (13 × 13 × 0.15 cm^3). We have found that this acrylamide:bis(acrylamide) ratio gives excellent resolution of nucleosomes in the monomer/dimer region. The buffer used to make the gel and circulate in the electrophoresis apparatus was that used by Todd & Garrard (1977), i.e., 6.4 mM Tris, 3.2 mM sodium acetate, and 0.32 mM EDTA, pH 8.0. Samples of ionic strength less than 5 mM were brought to 10% glycerol, 2 mM EDTA, and 0.025% bromphenol blue and loaded onto the gels. Sample loads contained 1–25 μg each of DNA and protein for one-dimensional slabs and 20–50 μg each of DNA and protein for one-dimensional tubes. Electrophoresis with recirculating buffer was for 15 min at 70 V followed by 4.5 h at 90 V for slab gels, and for 15 min at 50 V followed by 5.5 h at 60 V for tube gels. For visualization of DNA in the nucleoprotein samples, gels were stained with 1 μg/mL ethidium bromide in water for 0.5 h and photographed on an ultraviolet light box as before. For visuali-

zation of proteins, gels were stained in Coomassie blue R-250 or fluorographed.

Protamine-Release Method on Triton–Acid–Urea Gels. Electrophoresis of histone variants was performed at 25 °C with Triton–acid–urea (Cohen et al., 1975) slab gels containing 12% acrylamide [acrylamide:bis(acrylamide) 30:0.2], 8 M urea, 6 mM Triton X-100, and 5% acetic acid and polymerized in the presence of ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine (Temed). The plates were sealed with 15% acrylamide instead of agarose since many types of agarose interfere in the staining of the gels. Slab gels were 13 cm × 13 cm glass plates, and either 0.15 cm thick if used in the first dimension or 0.30 cm thick if used in the second dimension. Gels were preelectrophoresed for 5 h at 125 V with 5% acetic acid running buffer. The gel was then scavenged for 1.5 h at 125 V with 0.1 mL of a solution of 8 M urea, 0.3 M cysteamine, and 0.3 mg/mL protamine in each slot for one-dimensional slabs, or a total of 2 mL per gel in the second dimension. A second scavenge of 15–20-min duration was performed with a similar volume of 0.6 M cysteamine. The remaining scavenging solution was removed from the slots and the buffer replaced with fresh 5% acetic acid prior to applying the sample.

Analyses of micro samples of nucleosomes (<0.1 OD of nucleoprotein) were carried out on mini gels (3.25 × 4 × 0.031 in.^{3}) preelectrophoresed as described above with one-fifth the volume of scavenging solutions and for one-fifth the time (B. R. Shaw and W. M. Sholes, unpublished experiments). Histones in gradient fractions containing as little as 5 μg of total histones at 3 μg/mL concentration could be visualized on mini gels using the protamine-release procedure described below. Nucleoproteins in the sucrose-gradient fractions were precipitated overnight at −20 °C with 50 μg of protamine, 3 mM $MgCl_2$, and 2 volumes of ethanol (95%) and centrifuged at 16000*g* for 15 min. The pellet was dried by lyophilization, resuspended in 5 μL of Triton sample buffer (8 M urea–5% acetic acid–5% β-mercaptoethanol–2.5% thioglycolic acid–0.01% pyronin Y), and applied to the gel. Electrophoresis was at 210 V for 2 h.

Protamine release of histones from nuclear digests and concentrated nucleosome samples into Triton–acid–urea gels (Shaw & Richards, 1979) was accomplished as follows: samples of ionic strength less than 10 mM were made 8 M in urea, 5% in acetic acid, 2.5% in thioglycolic acid, 5% in β-mercaptoethanol (sample buffer), and 1.0% in protamine sulfate from Salmon sperm (histone free, Sigma) and applied directly to the gel. Electrophoresis (on 13 × 13 cm^2 gels) was for 8.5 h at 150 V. For two-dimensional electrophoresis to display histones, first-dimensional nucleoprotein tube gels (12 × 0.3 cm i.d.) were soaked for 0.5 h in 5% acetic acid, 5% β-mercaptoethanol, 2.5% thioglycolic acid, and 8 M urea. The tube was laid horizonally across the top of a preformed and prescavenged Triton slab (13 × 13 × 0.3 cm^3) with a 10-cm-long middle slot to hold the tube gel and a small slot at each end to serve as a sample well for a standard. The tube gel was carefully overlayered with 200 μL of a solution of 8 M urea, 5% acetic acid, and 1% protamine sulfate. Electrophoresis was for 13 h at 100 V.

Results

Chromatin Subunit Structure in the Two-Cell Embryo Exhibits a Nucleosome Structure. Figure 1A,B shows the electrophoretic migration of DNA extracted from nuclei isolated from two-cell embryos labeled with [^{3}H]thymidine during the first round of replication and digested with micrococcal nuclease. The photograph of the ethidium bromide stained

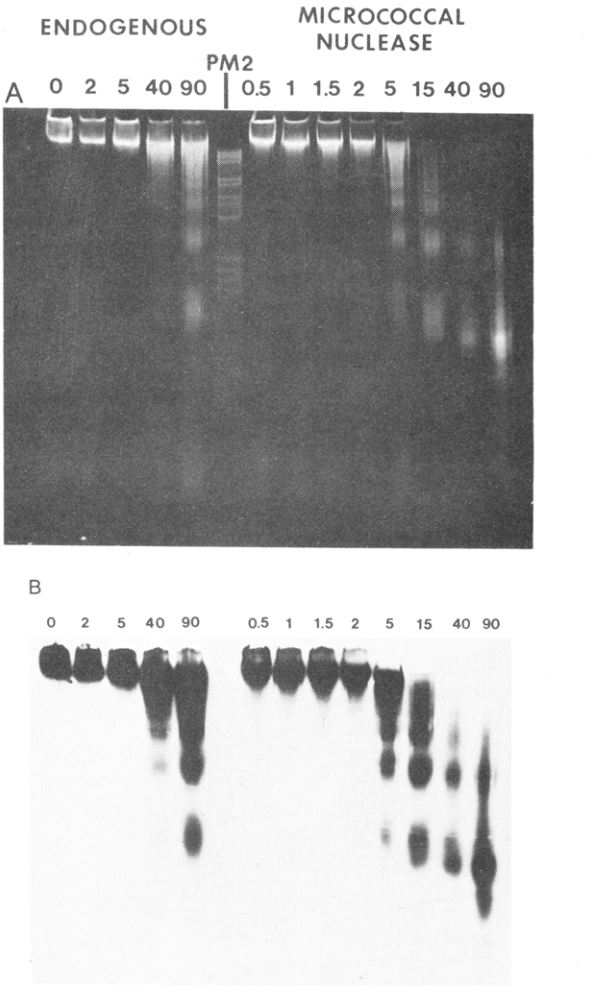

FIGURE 1: Electrophoretic patterns of ethidium bromide stained and tritium-labeled DNA fragments extracted from nuclei isolated from two-cell-stage embryos labeled (with 0.5 mCi/L [^{3}H]thymidine) for one generation beginning at fertilization. The nuclei were suspended in KPS buffer at 14 A_{260} units/mL and incubated in the presence of 1 mM $CaCl_2$ or digested with 10 units/mL micrococcal nuclease in the presence of 1 mM $CaCl_2$. The extracted DNA was electrophoresed on a 4% polyacrylamide gel. From left to right: nuclei incubated *without* nuclease at 37 °C for 0, 2, 5, 40, and 90 min, respectively; DNA restriction fragments of PM2-*Hae*III; same nuclei preparation incubated *with* micrococcal nuclease at 37 °C for 0.5, 1, 1.5, 2, 5, 15, 40, and 90 min, respectively. (A) Bulk DNA stained with ethidium bromide and photographed under UV light. (B) Same gel subjected to fluorography; the pattern corresponding to DNA synthesized over the course of the first cleavage.

gel (Figure 1A) and the fluorograph of the same gel (Figure 1B) are virtually superimposable in the monomer to multimer regions with a pattern typical of a nucleosomal structure, as expected from semiconservative replication. The DNA appears in discrete size ranges, and the patterns from the more extensively digested samples have dimer, trimer, and tetramer bands which migrate slightly faster than the corresponding bands in the less digested material. The zero-time control shows that there is no detectable low molecular weight DNA present in the two-cell nuclei before digestion with the nuclease. Samples to the left of PM2 show that an endogenous nuclease can be activated with $CaCl_2$ at 37 °C.

As with morula, blastula, and later stage chromatin (see following sections), the DNA in the two-cell embryo is readily accessible to nuclease. With both endogenous and exogenous nuclease treatment, the size of the nucleosomal repeat for two-cell embryos is ~225 base pairs (bp), or 10 bp larger than the 212 ± 5 bp repeat that we measure for blastula stage and

gastrula stage embryos (B. R. Shaw, G. Cognetti, and R. Rowland, unpublished experiments). The lack of DNA in the submonomer region when all high molecular weight DNA has been digested (Figure 1B) suggests that the core particle formed by the two-cell embryo is quite stable, as evidenced by its resistance to further digestion by micrococcal nuclease under these conditions. It also implies that the nucleoproteins and/or histones which comprise the core at the two-cell stage may be structurally similar to those found at later stages, since the core particle at later stages is also quite resistant to further degradation by micrococcal nuclease (not shown).

Further confirmation that the chromatin in the two-cell embryo exists in a nucleosomal structure can be seen in Figure 2A,B, where digested chromatin samples were loaded directly onto polyacrylamide gels and electrophoresed as nucleoprotein. In this experiment, embryos were labeled with [^{3}H]lysine during development. It can be seen that both the DNA (Figure 2A) *and* protein (Figure 2B) in the two-cell nucleoprotein fragments exhibit a typical nucleosome pattern. Furthermore, the monomer species from the two-cell stage migrate similarly to nucleosomes isolated from the blastula stage. One can also see evidence for both a 145-bp core particle and a 170-bp particle at the two-cell stage, providing further evidence that the chromatin structure in the two-cell embryo is similar to that of a fully developed system. This is significant since it implies that if chromatin remodeling is taking place from the haploid cell to the diploid cell (Albanese et al., 1980), it is essentially completed by this stage in the developmental scheme and is not, therefore, a gradual developmental process.

Thus, after one round of replication, the DNA is packaged into a nucleosomal-like structure in the two-cell embryo. Whereas previous studies on developing embryos have established that nucleosomes exist *after* differentiation, from this work we can conclude that nucleosomes also exist *prior* to differentiation, in cells which are "totipotent".

The determination of a chromatin structure for the two-cell embryo is noteworthy because the isolation of nuclei and chromatin from the two-cell stage sea urchin embryo is complicated by a number of factors. (1) Upon fertilization, the embryos rapidly form a protective sheath or fertilization envelope exterior to the outer membrane. Before cells can be isolated, this tough envelope must be removed or its hardening prevented by addition of inhibitors (*p*-aminobenzoic acid or aminotriazole). Both methods were employed here (see Materials and Methods) with similar results. (2) The nuclei from the two-cell stage are considerably more fragile than at any other stage of development and must be handled with extreme care. (3) The amounts of nuclear DNA and deoxyribonucleoproteins per culture are on the order of 500 times less than those found in a culture grown to swimming blastula stage. Therefore, in lieu of using massive cultures, we employed special analytical methods for isolating and studying chromatin structure of the two-cell embryo. (4) The cell is comprised predominantly of yolk proteins and nonnuclear nucleic acids such as RNA and mitochondrial DNA, the ratio of these extranuclear components to chromatin being very large. Contamination by these extranuclear components must be eliminated before one can examine the chromatin structure, particularly since the extranuclear components may contain phosphoprotein and phospholitin compounds which bind divalent ions, as well as large amounts of proteases and nucleases. If not removed from the nuclei preparation prior to nuclease digestion, extranuclear contaminants can prevent digestion entirely, or rapidly degrade the chromatin to give rise to

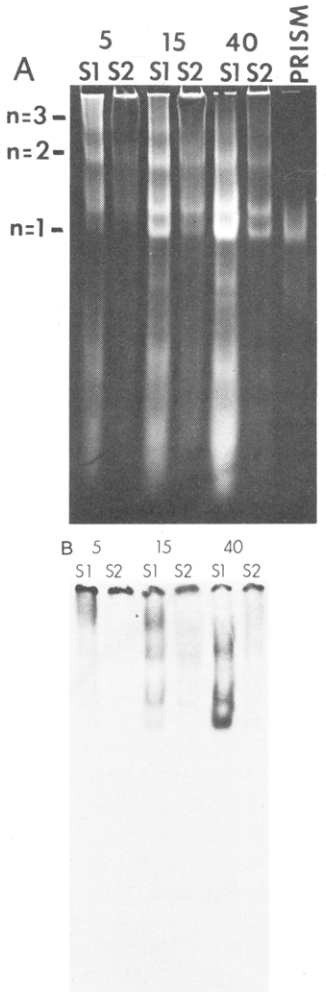

FIGURE 2: Electrophoretic separation of nucleosomes from the two-cell embryo. Embryos were labeled with [^{3}H]lysine (0.5 mCi/1.8 L) (80.5 Ci/mmol) after fertilization to the time of harvest, and nuclei were isolated (20 A_{260} units/mL) and digested with micrococcal nuclease (60 units/mL). For isolation of nucleosomes, a sample of the digestion mixture (nuclei in KPS buffer + 1 mM $CaCl_2$ + nuclease) was removed after 12 min, and EGTA was added to 10 mM to stop the digestion; the digestion mixture was centrifuged at 10 000 rpm for 15 min to give a supernatant (S1) and a pellet (P1). Both S1 and P1 were dialyzed at 4 °C for 24 h against three changes of low ionic strength buffer [10 mM Tris-HCl, pH 7.5 (25 °C), 0.1 mM EGTA, and 0.1 mM PMSF]. After dialysis, P1 was centrifuged at 10 000 rpm for 15 min to give a supernatant S2, and the S1 and S2 supernatants from three time points, 5, 15, and 40 min were electrophoresed on a 4% polyacrylamide gel. The ethidium bromide stained gel was photographed under UV light to reveal the nucleic acid (A), and subsequently fluorographed (B) for 1 month on preflashed film to visualize the protein labeled with [^{3}H]lysine during development. The sample labeled P was a monomer nucleosome fraction isolated from prism stage embryos.

spurious results (e.g., lack of dimer and larger nucleosomes, smeared nucleoprotein patterns, and/or ethidium bromide staining material in the monomer and submonomer regions of DNA and nucleoprotein gels). We have used the close correspondence of the digestion patterns of ethidium bromide stained and [^{3}H]lysine-labeled nucleoprotein, as in Figure 2A,B, as a means by which to test the cleanliness of our nuclei preparation and have concluded that the nucleosomal subunit structure can be obscured or completely obliterated if the nuclear preparation is contaminated.

Chromatin in the Eight-Cell Embryo Has a Typical Nucleosomal Structure; the Core Particles Are Composed of both CS and α-Type Histones. Having shown that the newly synthesized DNA is packaged into a nucleosomal structure

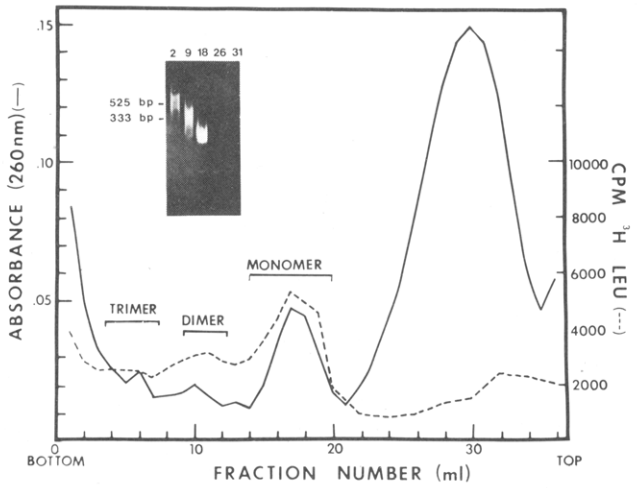

FIGURE 3: Isokinetic sucrose-gradient sedimentation profile of nucleosomes from eight-cell embryos labeled with [^{3}H]Leu during the first cleavage and chased with 10 mM leucine. The lysate from a 4-min micrococcal nuclease digest of the isolated nuclei was dialyzed against 2 mM Tris and 0.2 mM EDTA, pH 7.5, and fractionated on a 5–20% isokinetic sucrose gradient. Each gradient fraction was 1 mL. Inset: DNA extracted from the entire fraction (left to right, fraction numbers 2, 9, 18, 26, and 31) and analyzed on a 4% polyacrylamide slab gel.

in the two-cell embryo, it was important to determine whether both CS and α variant histone subtypes were constituents of the nucleosome core in early embryos. In this experiment, nuclei were isolated from embryos grown for one cleavage in the presence of [^{3}H]leucine, chased at the two-cell stage, and harvested at the eight-cell stage. When the DNA was extracted from a time-digest series and sized on polyacrylamide gels, it was seen to exhibit a discontinuous distribution of fragments that were integer multiples of a unit length, indicative of a nucleosome structure (data not shown).

The sucrose-gradient profile of the 4-min sample from this time-digest series is shown in Figure 3; the peaks correspond to monomer, dimer, and multimer nucleosomes. The size of the DNA in selected fractions is also shown (inset to Figure 3). The distribution of [^{3}H]leucine in the gradient corresponds closely to the absorbance profile of the DNA at 260 nm, except in the top third of the gradient which contains small fragments of DNA, a number of nonhistone proteins of low specific activity which are released by the digestion, and almost no histones (with the exception of a protein which migrates with the same mobility as CS H1).

Examination of proteins in the nucleosome fractions revealed that the CS proteins are found in nucleosomes of the eight-cell embryos, as can be seen most clearly in the H2A region of the Triton–acid–urea gel fluorogram (Figure 4B). Comparison of the H2A band intensities in the Coomassie-stained picture (Figure 4A) with the fluorogram (Figure 4B) suggests that the early embryos have a large pool of unlabeled CS H2A proteins (presumably derived from the egg) which are incorporated into nucleosomes of this stage. It can be noted in Figure 4 that the relative amounts of CS and α H2A histones in nucleosomes appear to be nearly constant across the gradient. On the Coomassie-stained gel, the CS H2A band in nucleosomes stains most predominantly whereas the ^{3}H-labeled CS and ^{3}H-labeled α H2A bands have approximately equal intensity on the fluorograph, as expected for a switching mechanism (Newrock et al., 1978a) in histone synthesis where CS H2A predominates through the four-cell stage after which α H2A synthesis is switched on. The smearing of the bands in this gel, which made it impossible to discriminate the H2B proteins, resulted because the low concentration of nucleo-

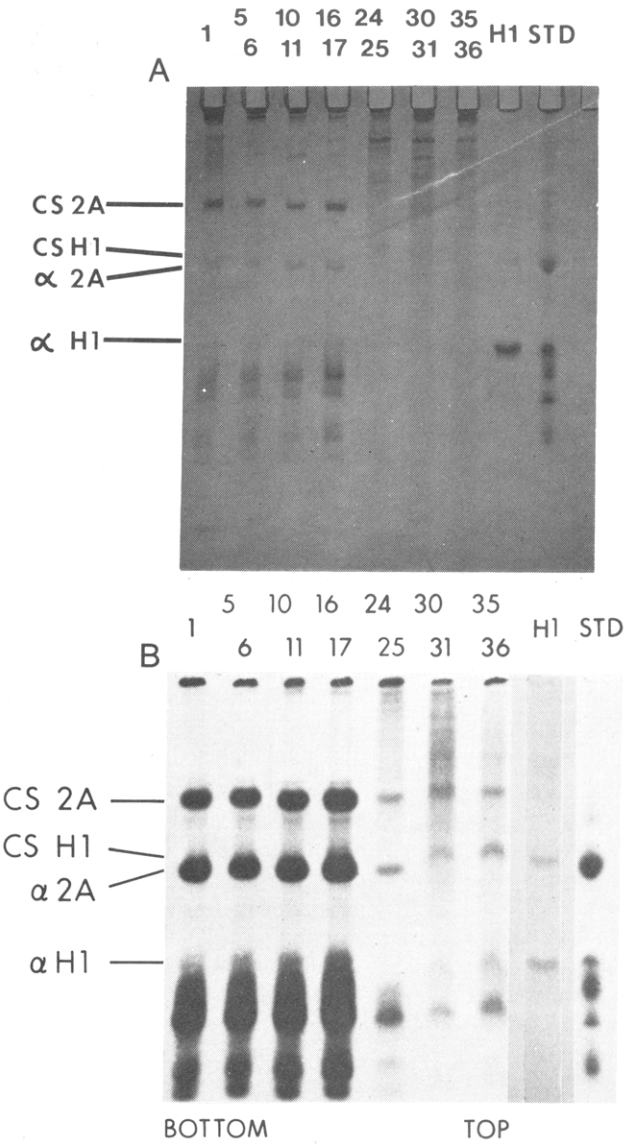

FIGURE 4: Electrophoretic analysis of the basic protein components of the pooled nucleosomes from eight-cell-stage embryos. The total nucleoprotein in pooled gradient fractions (left to right: 1, 5 + 6, 10 + 11, 16 + 17, 24 + 25, 30 + 31, and 35 + 36) of Figure 3 was precipitated with 2 volumes of ethanol in the presence of 50 μg of protamine and 3 mM $MgCl_2$ and subsequently electrophoresed on a 12% polyacrylamide–Triton–acid–urea mini slab gel using protamine release. Shown for comparison (far right) are total histones extracted from nuclei of the hatched blastula embryo (20 h) labeled with [^{3}H]leucine during the 10–20-h period. Histone H1 proteins were isolated by extraction with 5% perchloric acid from hatched blastula stage nuclei (labeled with [^{3}H]Leu from fertilization through the four-cell stage) which were extracted twice with 0.35 M NaCl. (A) Gel stained with Coomassie blue; (B) same gel after fluorography.

protein in the gradient fractions required large (2-mL) volumes to be processed into a 5-μL sample applied to each slot; CS H2B is found, however, in eight-cell-stage chromatin upon electrophoresis of whole nuclei using protamine to release the histones from the DNA (data not shown).

The near absence of core histones at the top of the gradient, the close correspondence of labeled protein and DNA across the bottom of the gradient, and the constant ratio of CS and α H2A histone across the gradient all indicate the following. (1) The CS proteins identified by Newrock et al. (1978a) are indeed nucleosomal proteins. They are incorporated soon after their synthesis into nucleosome structures, they can be isolated in nucleosomes by using standard methods for nucleosome preparation, and nucleosomes containing the CS protein sed-

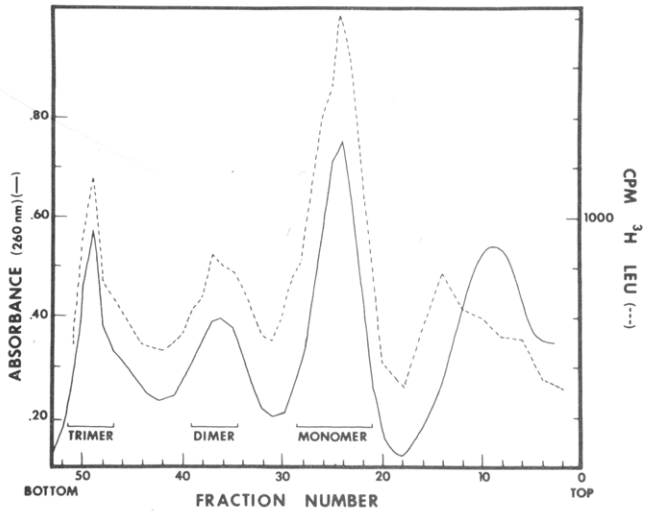

FIGURE 5: Isokinetic sucrose-gradient sedimentation profile of nucleosomes from hatched blastula embryos labeled with [^{3}H]Leu during the first cleavage and chased with 10 mM leucine. Nuclei were digested with 60 units/mL micrococcal nuclease in 0.3 M sucrose, 1 mM $CaCl_2$, and 10 mM Tris, pH 7.5, and the digestion was terminated with $^1/_9$ volume of 50 mM EGTA. The suspension was dialyzed overnight against 10 mM Tris, pH 7.5, 0.1 mM EGTA, and 0.1 mM PMSF, and the soluble lysate was fractionated on the gradient in a Spinco SW 27 rotor for 20 h at 26 500 rpm.

iment on sucrose gradients as expected for typical nucleosome structures. Their identification of the CS H1 protein as an H1-like protein which is synthesized during the first cleavages is consistent with the reported release of H1 proteins from digested nucleosomes after brief micrococcal nuclease treatment (Shaw et al., 1976a,b), and the release here of a protein (migrating with the same mobility as CS H1) into slower sedimenting material on the sucrose gradient. (2) Chromatin containing the CS histone variant of H2A appears to be about equally accessible to digestion with micrococcal nuclease as chromatin with α H2A, thus indicating that the subunit structures for both types of chromatin are rather similar. If mononucleosomes (or strings of nucleosomes having CS H2A) were more accessible to nuclease, we might expect to see a noticeable variation in the ratio of CS H2A to α H2A across the gradient; gross variations have not been observed.

Cleavage-Stage (CS) Proteins Are Found in Nucleosomes in the Hatching Blastula Stage. Although by blastulation the somatic α histones have replaced the CS proteins as the major proteins in chromatin, it seemed likely that the cleavage-stage histones would remain associated with the DNA *in a nucleosomal-type structure*, even after their synthesis ceased. To confirm this assumption, we labeled the developing embryos with [^{3}H]leucine from fertilization through the first two cleavages, and then they were grown to the hatching blastula stage without label.

The sucrose-gradient profile of a 30-min micrococcal nuclease treated lysate is shown in Figure 5. Note the similarities of both the DNA (A_{260}) and protein ([^{3}H]Leu) distributions in the regions where the monomers through multimers sediment. The protein in selected fractions from the gradient was analyzed on Triton–acid–urea mini gels, stained with Coomassie blue (Figure 6A), and subjected to fluorography (Figure 6B). With Coomassie stain (Figure 6A), the predominant histones in the H2A and H2B regions are the α-type histones, while the predominant histones detected with fluorography are those proteins synthesized during the first cleavages. Since the [^{3}H]Leu was incorporated about equally into the CS and α histones in a ratio of approximately 1:1, we conclude that the chase conditions were effective. Otherwise, most of the label would have ended up in α histones since it can be shown that a negligible amount of CS H2A is made after the 16-cell stage (Newrock et al., 1978a; B. R. Shaw, G. Cognetti, W. M. Sholes, and R. G. Richards, unpublished experiments). By comparing the stained gel (Figure 6A) with the fluorogram (Figure 6B), one can see that the H2A and H2B histones synthesized during the first two cleavages are found in nucleosomes isolated from blastulated embryos and that the relative amounts of CS H2A and α H2A found in different size nucleosomes remain constant across the gradient. Furthermore, the amount of cleavage-stage histone H2A found at different densities in the sucrose gradient is that expected for a fractionation of nucleosomes; the histone concentration correlates well with the DNA concentration in the nucleosomal fractions. We also found no cleavage-stage or α-histone variants in the top one-third of the gradient. The correlation of the histone and DNA patterns in the bottom two-thirds of the gradient, as well as the lack of histones in the top two-thirds of the gradient, is representative of the behavior expected for nucleosomes. We, therefore, conclude that nucleosomes at the hatching blastula stage (after ten divisions) contain histones that are synthesized by the two-

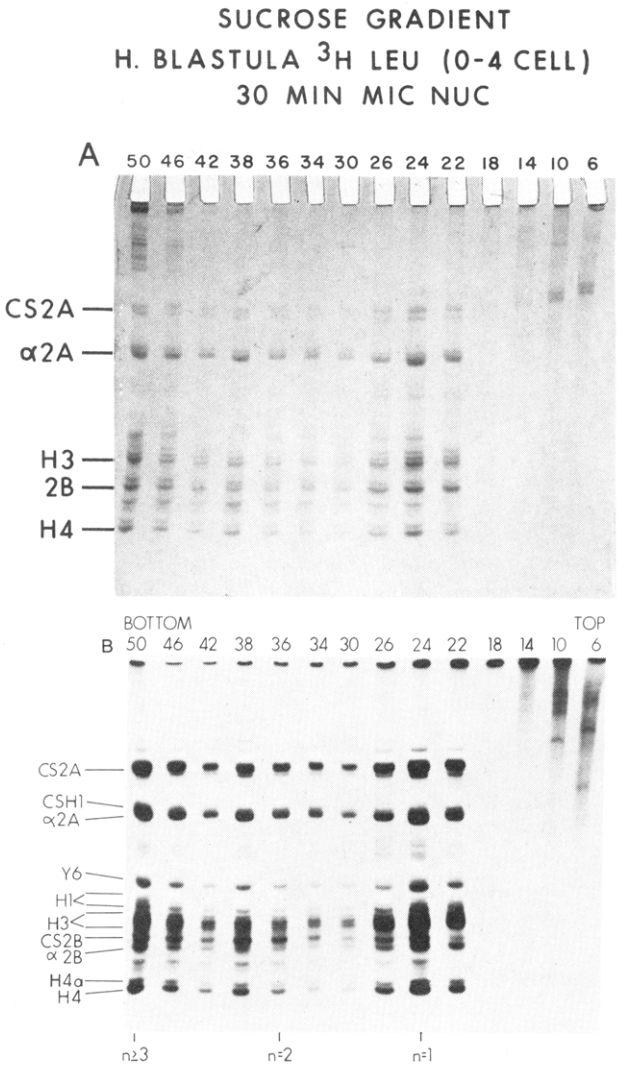

FIGURE 6: Electrophoretic analysis of basic proteins of nucleosomes from hatched blastula embryos labeled with [^{3}H]Leu at fertilization and chased at the 4-cell stage. Aliquots (200 μL) of selected fractions from the gradient in Figure 5 were processed as described in Figure 4 and electrophoresed on a Triton–acid–urea mini gel using the protamine-release method. (A) Gel stained with Coomassie blue; (B) same gel after fluorography.

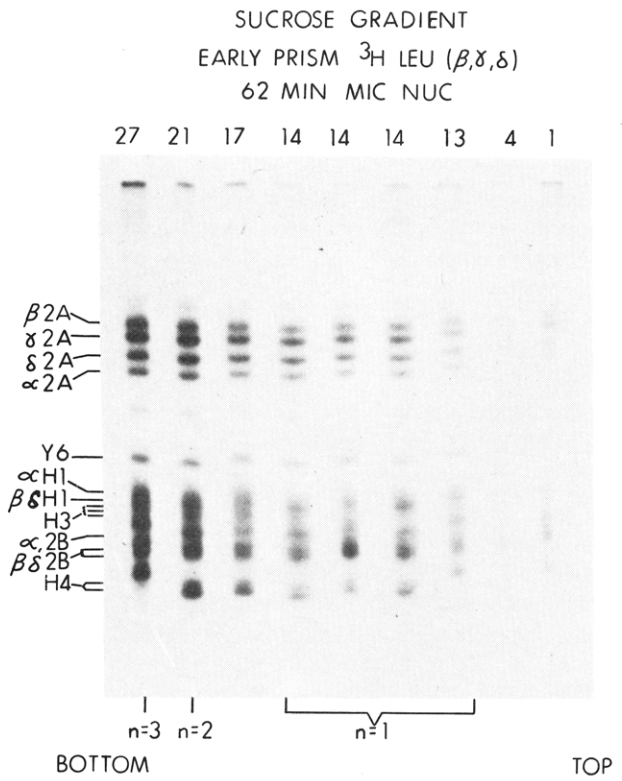

FIGURE 7: Electrophoretic analysis of proteins of nucleosomes from embryos isolated at early prism stage labeled with [^{3}H]Leu from gastrula stage to harvest. Only the fluorogram is shown. The two slots at the right are samples from the top of the sucrose gradient which reveal no histones. Monomer nucleosomes, n = 1; dimers, n = 2; trimers, n = 3.

and four-cell-stage embryos, as well as histones synthesized at later stages. Such results imply the existence of different classes of nucleosomes, heterogeneous in their content of histone variants.

Putative H2A β, γ, and δ Subtypes Replace the H2A α Histone in Late-Stage Nucleosomes. One general question about the identity of the other H2A subtypes remains: how certain can we be that each of the heterogeneous late-stage putative β, γ, and δ H2A proteins detected on Triton–acid–urea gels is, in fact, an authentic histone subtype? To date, the evidence is only indirect, since no sequence or tryptic mapping data have been reported for these putative H2A β, γ, and δ proteins. Each of the putative H2A β, γ, and δ proteins is encoded by a distinct and separable mRNA (Kunkel & Weinberg, 1978; Childs et al., 1979) and migrates on $NaDodSO_4$ gels with the approximate mobility of α H2A and α H2B (which are poorly resolved in such gels). We reasoned that if these late proteins are in fact histone H2A subtypes, then each should be found in nucleosomes and at late stages these putative subtypes should replace the α H2A subtypes in the core particle. By late gastrula and early prism stages, the predominant functional and radioactive β, γ, and δ H2A histones found in the nucleosome should correspond to the H2A β, γ, and δ forms.

Experimental verification of this fact is found in Figure 7. Embryos were labeled at the mesenchyme blastula stage with [^{3}H]Leu and grown to early prism stage. Nuclei were prepared and treated with micrococcal nuclease. Nucleosomes from the prism stage nuclei were separated on an isokinetic sucrose gradient, and the proteins were analyzed on Triton–acid–urea gels by the protamine-release electrophoresis method. In the Coomassie-stained Triton–acid–urea gel (not shown), the α H2A species predominates in the monomer, dimer, and multimer nucleosomes, reflecting the approximate relative stoichiometrical amounts of the H2A variants found in chromatin at early prism stage. Figure 7 (an autofluorogram of the same gel) shows the labeled proteins in monomer, dimer, and multimeric nucleosomes. Since the embryos were labeled with [^{3}H]Leu at the mesenchyme blastula and collected at the early prism stage, only histones synthesized during the last one or two divisions would be labeled. Most of the tritium which is found in the nucleosomes ends up in the β, γ, and δ H2A, rather than in the α H2A histone. Such experiments suggest a *minimal* level of translational control of H2A at the later stages of development. Important for this work is that the total H2A messenger RNA activities observed by Childs et al. (1979) and Kunkel & Weinberg (1978) correspond closely with the type and amount of histone H2A products we find in nucleosomes from the prism stage. The majority of [^{3}H]Leu-labeled protein ends up in the bottom two-thirds of this gradient; as expected for proteins which are involved in a nucleosomal structure, almost none of the α and putative β, γ, and δ H2A proteins are found in the upper third of the gradient. Furthermore, the relative amounts of α, β, γ, and δ H2A histones found in different size nucleosomes across the gradient also remain constant.

In these studies, we observed that the amount of α H2A protein [which was the histone most predominantly stained and labeled at blastula stage (see Figure 6)] decreased until prism stage when the relative amount of newly synthesized β, γ, and δ H2A histone found in the nucleosomes exceeded the amount of α H2A. For comparison, the relative amounts of H2A β, γ, and δ proteins found in nucleosomes at the late gastrula stage correspond to that which is translated in a cell-free translation system from whole embryo RNA (Childs et al., 1979). Such experiments suggest a minimal level of translational control of H2A at later stages and confirm that the β, γ, and δ H2A proteins are associated with nucleosomes.

Discussion

We have isolated monomer and multimer nucleosomes from five stages in the early development of *Strongylocentrotus purpuratus* sea urchin embryos and analyzed the respective nucleosome populations for their distribution of histone variants on Triton–acid–urea gels using our protamine-release technique. This study was possible since our protamine-release method allows for the analysis of histone variants in fractions of nucleosomes having as little as 5 μg of nucleoprotein/mL.

Our results indicate that 11S monomer and multimer nucleosomes *can* be isolated from sea urchin nuclei at all stages of early development, from the two-cell embryo through the prism stage. From the 8-cell stage to the prism stage, we observe that the predominant histone composition of the monomer nucleosome closely parallels the total histone population of the cell. Histones which are synthesized immediately after fertilization by the cleavage-stage embryo will readily assemble onto DNA as nucleosomes, and cleavage stage histones which originate from the egg are also found in nucleosomes. Furthermore, although their synthesis ceases in morula (Cohen et al., 1975), the cleavage-stage histone variants appear to be retained within the nucleosomal structure of chromatin from the time of their synthesis through the prism stage of development. All five variants (CS, α, β, γ, and δ) of histone H2A that are synthesized subsequent to fertilization [those identified by Cohen et al. (1975) in prism stage nuclei] are found as components of mononucleosomes isolated from cells at the prism stage.

These results allow insight into the assembly processes of nucleosomes and imply that nucleosomes at the prism stage

must consist of a minimum of five distinct subpopulations corresponding to the five subclasses (CS, α, β, γ, and δ) of histone variants [assuming total and conservative segregation of newly synthesized histones to one of the newly synthesized daughter duplexes as proposed by Leffak et al. (1977)]. In addition, these results rule out the possibility that the histone variants found in early stages of development might be the products of nonsense mutations within some of the repetitive histone gene sequences during the process of evolution. If segregation of histones to DNA during replication is nonconservative, the increased number of permutations greatly increases the types of microheterogeneous nucleosomes. These findings demonstrate the validity of the hypothesis of microheterogeneous nucleosomes as predicted by Cohen et al. (1975).

From our studies of nucleosomes, it would appear that the "cleavage-stage" proteins are histones and that both the maternally derived and newly synthesized cleavage-stage histones associate with DNA immediately to form nucleosomes (unpublished experiments). This association, once established, is a rather stable structure since the nucleosomes isolated from the blastula and prism stage also contain early synthesized cleavage-stage histones in substantial amounts. These results confirm predictions postulating the existence of multiple forms of nucleosomes on the basis of data which showed that early synthesized histone subtypes remain associated with chromatin even after some of them cease to be synthesized.

Thus, corresponding to the well-known switch in histone mRNA population and a shift in transcribed histone protein products observed during embryogenesis in the sea urchin, these studies provide evidence for a shift in the main nucleosomal population of the cell during embryogenesis.

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