Temporal Changes in Nucleosome Populations during Sea Urchin Early Development†

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ABSTRACT: The finding that the major H2A histone isolated in nucleosomes of a particular developmental stage corresponds to the major H2A subtype synthesized at and prior to that stage [Shaw, B. R., Cognetti, G., Sholes, W. M., & Richards, R. G. (1981) *Biochemistry 20*, 4971–4978] suggests that the nucleosome population of *Strongylocentrotus purpuratus* sea urchin embryos becomes increasingly heterogeneous through development. Nuclei from morula, blastula, and prism stages were digested with micrococcal nuclease and the nucleosomes fractionated by their solubility at different ionic strengths (≤0.1 M NaCl). Comparison of the nucleoproteins in the resulting fractions by polyacrylamide gel electrophoresis revealed trends of decreasing solubility and increasing nucleoprotein heterogeneity through development, which paralleled programmed developmental switches in histones. Whereas morula- and blastula-stage chromatins digested to two distinct and relatively stable monomer nucleosome species, prism-stage chromatin exhibited broad heterogeneity in the monomer nucleosome region. Two-dimensional analysis of proteins from blastula-stage nucleosomes indicated an asymmetric distribution of histone H1 and minor amounts of non-histone proteins associated with dimer nucleosome subsets, which were also characterized by differences in their relative mobilities and proportions of H2Aα and H2Bα histones. These results suggested that variations in core histone subtypes may promote nucleosome conformational heterogeneity.

Switches in variant histone synthesis during development have been well characterized for the sea urchin *Strongylocentrotus purpuratus* (Cohen et al., 1975; Newrock et al., 1978a,b; Kunkel & Weinberg, 1978). Cleavage-stage (CS) histones, synthesized from maternal messenger RNA stored in the egg (Salik et al., 1981; Poccia et al., 1981), are the predominant H2A and H2B subtypes synthesized during the first embryonic division. By the morula (~64-cell) stage of development, cleavage-stage histone synthesis is substantially reduced, and the α-histone subtypes of H2A and H2B are the major forms observed. The α-histones are synthesized predominantly from embryonic messenger RNA (Weinberg et al., 1983; Maxson & Wilt, 1982). By blastula (~300-cell) stage, synthesis of cleavage-stage histones is shut down, while synthesis of α-histone subtypes continues. By this point, synthesis of the late or β, γ, and δ subtypes of histone H2A as well as the γ and δ (late) subtypes of histone H2B is switched on (there appears to be no β subtype for histone H2B). In contrast to the cleavage-stage and α-histone variants, which are at least in part transcribed from maternal messenger RNA, the β, γ, and δ variants of H2A and H2B are transcribed from embryonic messenger RNA. At later stages in development (prism and pluteus), these subtypes are the predominant forms of the H2A and H2B histone classes. Additional histone variants derived from sperm chromatin may also be present in minor amounts at early developmental stages (Poccia et al., 1981; Strickland et al., 1978). Histones of a particular class differ by one or several amino acid residues in their primary sequence and can be identified by electrophoresis in (Triton) acid/urea polyacrylamide gels (Cohen et al., 1975).

In 1981, Shaw et al. reported that the H2A subtypes found in nucleosomes isolated during early developmental stages of the sea urchin embryo correspond to the stage-specific shift in histone synthesis observed by Cohen et al. (1975), thus establishing that all of the proposed H2A variants and probably the H2B variants function as proteins of the nucleosomal core (Shaw et al., 1976). These histone variants are retained in the nucleosomal chromatin matrix as development proceeds (Cohen et al., 1975; Shaw et al., 1981). Thus, CS histones which are synthesized primarily during early cleavage (one- to eight-cell stage) are observed in nucleosomes isolated from blastula-stage embryos. Because 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., 1978b), 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 might be correlated with switches in variant histone synthesis.

We have initiated experiments to determine whether nucleosomes isolated at different stages in development have unique properties which may be correlated to the observed shift in nucleosomal proteins discussed above. The results of the experiments, reported here, indicate that there are differences in nucleosomes isolated at different developmental stages, as evidenced by their solubility properties and their electrophoretic migration in polyacrylamide gels. Small differences in the H2A histone population of different nucleosomes further suggest that histone variants may be linked (either directly or indirectly) to the differences observed.

Experimental Procedures

Isolation of Gametes and Culture of Embryos. Strongylocentrotus purpuratus sea urchin embryos were obtained from the Pacific Biomarine Laboratory, Venice, CA. Gametes were harvested by coelomic injection with 0.55 M KCl. The eggs were filtered through four layers of cheesecloth and then settled 2–3 times in artificial seawater and fertilized (~95%). The embryos were grown to the appropriate stage at a concentration of 3–4 mL of packed eggs per L of artificial seawater at 16–17 °C. Embryos that were harvested prior to blastu-

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lation were grown in seawater containing "hatching enzyme" which was collected from an equivalent culture of hatched blastula-stage embryos and filtered prior to use. When used within 24 h (stored at 5 °C), this media facilitated removal of the fertilization envelope. The artificial seawater used in these experiments was 4.46×10^{-1} M NaCl, 2.55×10^{-2} M $MgCl_2 \cdot 6H_2O$, 1.82×10^{-2} M $MgSO_4 \cdot 7H_2O$, 9.26×10^{-3} M KCl, 2.36×10^{-1} M $NaHCO_3$, and 4.91×10^{-3} M $CaCl_2 \cdot 2H_2O$ (Tyler, 1953).

Nuclei Isolation and Digestion. S. purpuratus embryos at the appropriate stage of development were harvested by straining through a 52-μm mesh Nytex filter (Tectonics) and rinsed twice with artificial seawater, twice with 0.8 M dextrose/0.1 M NaCl, and once with 0.2 M dextrose/0.15 M NaCl. Embryos were concentrated by centrifugation at 500 rpm for 3 min (Sorvall HB-4). The pelleted embryos were then brought up in A1 buffer + 1.0 mM phenylmethanesulfonyl fluoride (PMSF) [A1 buffer: 60 mM KCl, 15 mM NaCl, 0.15 mM spermidine, 0.5 mM spermine, 15 mM β-mercaptoethanol, 15 mM tris(hydroxymethyl)aminomethane (Tris), 0.34 M sucrose, 2 mM ethylenediaminetetraacetic acid (disodium salt) (EDTA), and 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), pH 7.4] (Marshall & Burgoyne, 1976) and lysed by forced passage through a 30-μm mesh Nytex filter (Tectonics); 20–25 passages were usually necessary for blastula- and prism-stage embryos. The broken cell suspension was then filtered through a 10-μm mesh Nytex filter (Tectonics) to retain large cellular debris and unbroken cells, and the filtrate was layered over A2 buffer (same as A1, but with 1.37 M sucrose, 1 mM EDTA, and 0.3 mM EGTA) (filtrate volume:A2 volume, 3:1) and then centrifuged at 10000 rpm for 10 min (Sorvall HB-4). The pellets were initially resuspended in A4 buffer (same as A1, but with 0.34 M sucrose, 0.1 mM EDTA, and no EGTA) to which an equal volume of A4 buffer with 0.25% NP-40 was added. After 5 min of incubation at 5 °C, the nuclei were pelleted at 4000 rpm for 10 min (Sorvall HB-4). The nuclear pellet was then carefully resuspended in NP-40-free A4 buffer and centrifuged as before. This step was sometimes repeated once more, and the pelleted nuclei were carefully resuspended (80–100 A_{260} units/mL) in A4 buffer supplemented with 1 mM $CaCl_2$ for digestion with micrococcal nuclease. All micrococcal nuclease (Worthington) digestions were carried out at 37 °C at a concentration of 125 units/mL and 1.25 units/A_{260}. Aliquots were removed from the digestion mixture at 0, 2, 5, 12, 30, and 80 min, and digestion was terminated by adjusting the mixtures to 3 mM EDTA with a 100 mM stock solution. The morula-stage samples were dialyzed vs. 2 mM Tris and 0.2 mM EDTA, pH 7.75, and centrifuged for 10 min at 5000*g* to yield a supernatant (S) and pellet (P) which were then electrophoresed in a 4% low ionic strength polyacrylamide gel. Although nearly all of the nucleosomes at morula stage were found in the supernatant, this was not true for blastula- and prism-stage nuclei. The latter were subjected to the salt fractionation described below.

Salt Fractionation: Modified Method of Levy W. & Dixon (1978). All operations were carried out at 0–5 °C. Blastula- and prism-stage nuclei digestion mixtures of 300-μL volume (at an approximate concentration of 80 A_{260} units/mL) in 1.5-mL Eppendorf tubes were centrifuged at 6000 rpm for 15 min (Sorvall SS-34). After the supernatants (S1) were removed, the pellets (P1) were suspended in 100 μL of 2 mM Tris and 0.2 mM EDTA, pH 7.75 (pH measured at 25 °C). The S1 supernatants and P1 pellets were then dialyzed vs. 2 mM Tris and 0.2 mM EDTA, pH 7.75. S1 supernatants were saved for analysis; the dialyzed P1 pellets were centrifuged at 17000 rpm for 30 min (Sorvall SS-34) and the supernatants (S2) removed. These pellets (P2) were suspended in 75 μL of 2 mM Tris and 0.2 mM EDTA, pH 7.75, and saved for analysis. The S2 supernatants were made 0.1 M in NaCl by adding $^1/_9$th volume of 1.0 M NaCl and allowed to incubate at 0–5 °C. After 30 min, they were centrifuged at 12000 rpm for 30 min; the supernatants (S3) were removed and dialyzed vs. 2 mM Tris and 0.2 mM EDTA, pH 7.75. The corresponding pellets (P3) were suspended in 50 μL of dialysis buffer and dialyzed vs. the same medium. This procedure yielded four final fractions, S1, P2, S3, and P3.

Electrophoresis. Electrophoretic analysis of DNA in the first and second dimension was carried out at 25 °C in 4% polyacrylamide gels according to Loening (1967) supplemented with 0.1% sodium dodecyl sulfate (SDS) as described by Todd & Garrard (1977). Electrophoresis of nucleoproteins in 4.0% polyacrylamide gels was performed in the presence of 0.32 mM EDTA, 3.2 mM sodium acetate, and 6.4 mM Tris, pH 8.0 (Todd & Garrard, 1977), as previously described (Shaw & Richards, 1979; Shaw et al., 1981).

Analysis of proteins with the in situ protamine-release method into (Triton) acid/urea gels was described previously (Shaw et al., 1981; Richards & Shaw, 1982). Briefly, nucleoprotein samples of ionic strength <0.1 M and an initial concentration of 50–60 A_{260} units/mL were adjusted to 8 M urea, 5% acetic acid, 2.5% thioglycolic acid, 5% β-mercaptoethanol, 0.01% pyronin Y, and 1.0% protamine (w/v) (termed "sample buffer") by adding 2 volumes of 1.5× sample buffer and were then applied directly to a prescavenged 12% acrylamide gel [30:0.2 acrylamide:bis(acrylamide)] containing 8 M urea, 6 mM Triton X-100, and 5% acetic acid (Cohen et al., 1975; Zweidler, 1978). Incubation times of 5 min at 25 °C were sufficient for well-dispersed samples. Electrophoresis in 13 × 13 × 0.15 cm^3 slab gels was routinely carried out for 8.5 h at 150 V. For two-dimensional electrophoresis to display histones, first-dimension nucleoprotein tube gels (12 cm × 0.3 cm i.d.) were soaked for 0.5 h in sample buffer without protamine. The tube was laid horizontally across the top of a preformed and prescavenged (Triton) acid/urea slab gel (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. After the buffer chambers were filled with fresh 5% acetic acid, the tube gel was carefully overlayered with 200 μL of 5% acetic acid, 8 M urea, 1.0% protamine, and 0.01% pyronin Y. Electrophoresis was carried out for 13 h at 100 V. The proteins were stained with Coomassie blue R-250 by the 2-propanol procedure of Fairbanks et al. (1971) and then photographed and scanned.

Gel Scanning. Proteins and nucleoproteins in polyacrylamide gels were scanned by using a GCA/McPherson ultraviolet–visible (UV–Vis) spectrophotometer with a scanning attachment. Polyacrylamide gels stained with Coomassie blue were scanned in a glass boat (15 cm long) at 546 nm. Polyacrylamide gels containing nucleoproteins were scanned in a quartz boat at 260 nm.

Results

The increase in nucleosomal DNA repeat length through development (Arceci & Gross, 1980; Savic et al., 1981; Chambers et al., 1983) is most likely a reflection of altered protein–DNA interactions resulting from the sequential introduction of different histone variants through development or from changes in the non-histone protein population. One would expect that such differences in chromatin structure through development might lead to observable differences in

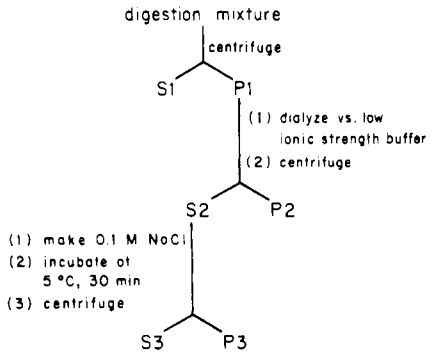

FIGURE 1: Diagram of the nucleoprotein fractionation scheme [modified from Levy W. & Dixon (1978)]. The low ionic strength buffer was 2 mM Tris and 0.2 mM EDTA, pH 7.75. Centrifugation was for 10 min at 5000*g*.

the type and solubility properties of nucleosomes isolated at different stages of development. Therefore, micrococcal nuclease time-digest samples of morula-, blastula-, and prism-stage nuclei were subjected to fractionation, as described below.

The time-digest samples obtained for the blastula and prism stages were fractionated by a modified procedure of Levy W. & Dixon (1978) to yield four final fractions, S1, P2, S3, and P3 (Figure 1). These fractions were (S1) nucleoproteins which were soluble in the digestion mixture, (P2) nucleoproteins which were insoluble in the digestion mixture and remained insoluble after dialysis vs. a low ionic strength buffer, (S3) nucleoproteins which were insoluble in the digestion mixture but soluble after dialysis vs. a low ionic strength buffer and soluble after addition of NaCl to 0.1 M, and, finally, (P3) nucleoproteins which were insoluble in the digestion mixture but soluble after dialysis vs. a low ionic strength buffer and insoluble after addition of NaCl to 0.1 M. Because the amount of nuclei obtained at morula stage was much less than that at blastula and prism stages, an abbreviated fractionation procedure was employed; these time-digest samples were simply dialyzed vs. a low ionic strength buffer and then centrifuged. The resulting supernatant (S) represented all of the morula-stage nucleoprotein that was soluble at low ionic strength and was therefore about equivalent to the S2 fraction (i.e., S3 + P3) from blastula and prism stages. Similarly, the resulting pellet (P) was similar to, but not identical with, the P2 fraction of blastula and prism stages in that it represented all material insoluble at low ionic strength.

After fractionation, salt-containing samples were dialyzed vs. low ionic strength buffer, and the nucleoproteins from all fractions were separated by polyacrylamide gel electrophoresis (Figure 2). Since the samples were loaded onto the gels in amounts equivalent to the proportion of material in each fraction, the ethidium bromide staining intensity could be used to estimate the relative distribution of nucleoproteins among the various fractions at each time point.

At morula stage, nearly all the small oligonucleosomes were soluble in low ionic strength buffer (S fraction), a property unique to chromatin from this stage. Only a very small proportion of nucleoprotein was seen in the pellet even at early times in digestion; this nucleoprotein did not enter the gel and disappeared with increased digestion (cf. Figure 2). At blastula stage, the majority of nucleoprotein was also found to be soluble at low ionic strength (S3 + P3) (similar to morula stage); however, a significantly greater amount of nucleoprotein remained low ionic strength insoluble (P2). In contrast, at prism stage, few nucleoproteins were soluble at low ionic strength S2 (i.e., S3 + P3 fractions), while the majority of nucleoprotein was seen to be insoluble at low ionic strength (P2). Thus, there appeared to be a trend of decreasing nucleoprotein solubility as development proceeded from morula through prism stages.

The possibility existed that, in dialyzing the nucleoproteins to low ionic strength for separation in polyacrylamide gels, some of the nucleoproteins might have become insoluble with changes in salt concentration and thus not have entered the gel. To check this, we analyzed the DNA and protein contents of all the prism-stage fractions, wherein the greatest insolubilities were observed. The total DNA and protein contents in each fraction (shown in Figure 3) closely approximated the amounts seen on the nucleoprotein gels (cf. Figure 2, prism stage). Had most of the nucleoprotein become insoluble and not been able to enter the nucleoprotein gel, one would have expected to observe more DNA and/or protein (especially in the P2 fraction) by component analysis than that expected from the nucleoprotein gel. This is apparently not the case.

Differences were observed not only in the nucleoprotein solubility at successive stages in development but also in the classes of nucleosomes generated by micrococcal nuclease digestion. We found that nucleoprotein from early stage embryos (morula) migrated as two discrete monomer bands and one slightly diffuse dimer band (Figure 2). Nucleoprotein from blastula-stage embryos migrated either as two discrete monomer bands and two discrete dimer bands or as a heterogeneous mixture of monomers, particularly noticeable in the S3 fractions. In general, the discrete dimers became more prominent and had less tailing at later times of digestion. Later stage (prism) nucleoprotein, on the other hand, migrated only as broad, diffuse bands in both the monomer and dimer nucleosomes in all of the fractions, and resembled the electrophoretic migration pattern of the DNA component, above (Figure 3). Furthermore, digestion directly to nucleosomes migrating with the mobility of cores (i.e., fast migrating nucleosomes) seemed to occur at this stage, in contrast to the earlier stages where a relatively stable mononucleosome species (migrating behind the core) predominated in addition to the nucleosome core (Figure 2). This trend was true for all times of digestion and for all nucleoprotein fractions at the prism stage of development. Thus, not only were there fundamental differences in the types and solubility behavior of nucleoproteins from the different stages of development but also there appeared to be an increasing variety of monomer and dimer nucleosomes as development proceeded from the morula through the prism stage.

Since this increasing microheterogeneity correlated well with the addition of histone subtypes to the chromatin matrix during development (Cohen et al., 1975; Shaw et al., 1981), we undertook a more extensive analysis to determine if there was any correlation between protein composition and nucleosome migration in polyacrylamide gels. We reasoned that a salt-fractionated sample would be a less complex system to study than a whole nuclease digest and that enrichment of particular histone variants or non-histone proteins might be observed in the electrophoretically separated nucleosomes of such a sample. Since early and late variants are synthesized at blastula stage, we therefore chose the nucleoproteins of the blastula-stage 5-min P3 fraction (shown in Figure 2) for this study. Separation of these nucleoproteins in two 4% low ionic strength polyacrylamide tube gels provided the first-dimensional separation for subsequent two-dimensional analysis of either proteins or DNA. As seen by the close correlation between the A_{260} profile of DNA migrating as nucleoproteins in the tube gel of Figure 4 and the ethidium bromide stained nucleoprotein in the slab gel (Figure 2; blastula-stage 5-min P3),

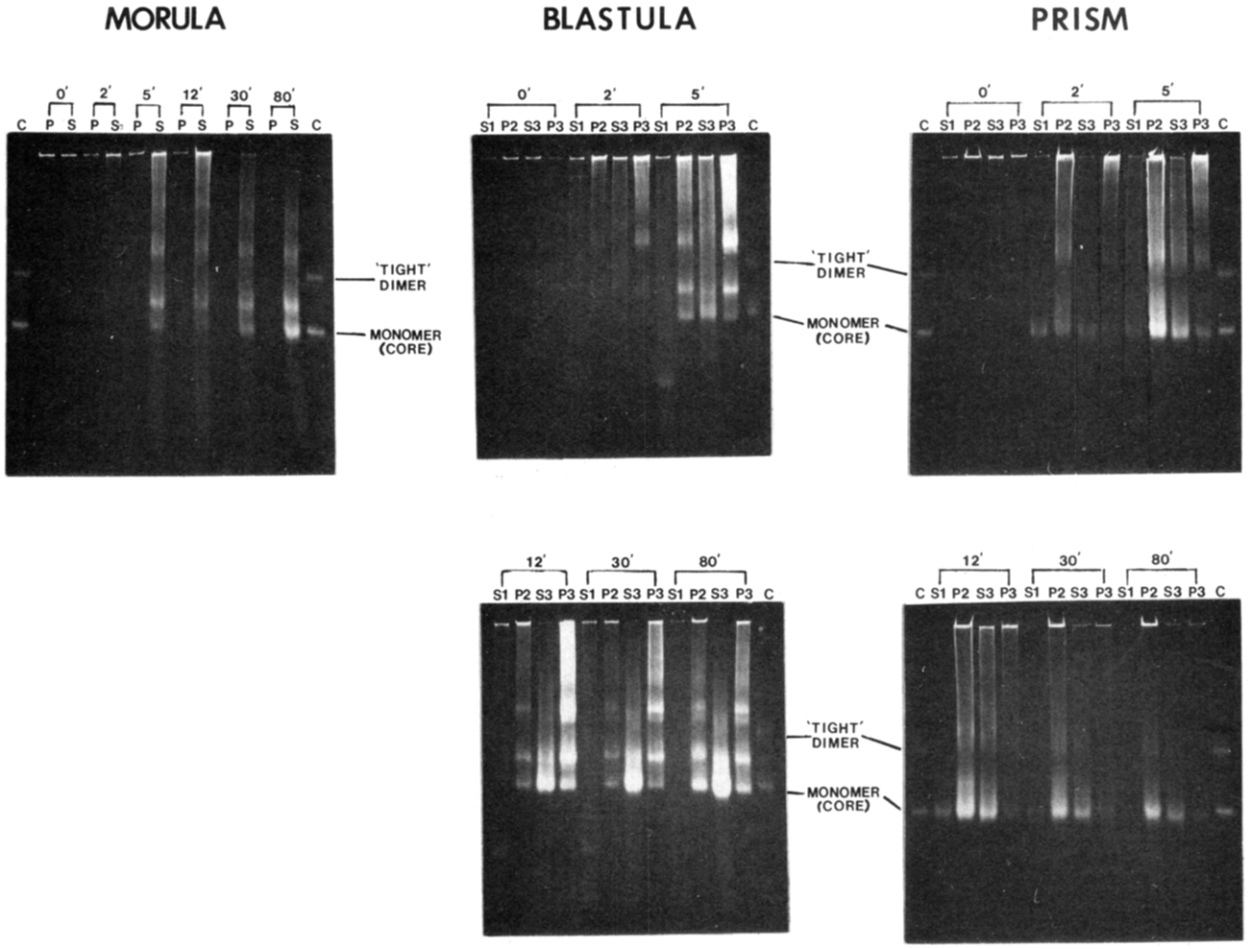

FIGURE 2: Electrophoretic separation of morula-, blastula-, and prism-stage nucleoproteins on 4% low ionic strength polyacrylamide gels. Isolated nuclei (100 A_{260} units/mL) from the respective stages were digested with micrococcal nuclease (125 units/mL) for 0, 2, 5, 12, 30, and 80 min in the presence of 1 mM $CaCl_2$; digestion was terminated by the addition of 100 mM EDTA to a final concentration of 3 mM. Morula-stage digestion mixtures were dialyzed vs. 2 mM Tris and 0.2 mM EDTA, pH 7.75, and then centrifuged to yield a supernatant (S) and pellet (P). Blastula- and prism-stage nuclei were fractionated by the protocol shown in Figure 1 to yield four fractions: S1 (digestion supernatant), P2 (low ionic strength insoluble), S3 (0.1 M NaCl soluble), and P3 (0.1 M NaCl insoluble). These fractions were then dialyzed vs. 2 mM Tris and 0.2 mM EDTA, pH 7.75, prior to electrophoresis. The volume of sample from each fraction which was loaded onto the gel was determined by calculating the volume containing 1 A_{260} unit of nucleoprotein if all nucleoprotein had fractionated to that fraction. (A correction was made for changes in volume during dialysis.) The amount of sample applied was, therefore, representative of the relative proportions of nucleoprotein in each fraction. The gels were stained with ethidium bromide. A preparation of core and "tight-dimer" nucleoproteins isolated from chicken erythrocytes (C) was also electrophoresed for comparison. The "tight-dimer" contains two histone octamers, but the DNA fragment is shorter than that of the normal dimer [see Shaw & Richards (1979)].

the nucleoprotein migration was comparable in the two gels. In particular, both gels revealed a faint core and a prominent monomer. In the dimer region of the A_{260} profile for the tube gel, three or more distinct nucleoprotein species could be distinguished, and in the oligomer region, there appeared to be at least seven distinguishable nucleoprotein species which were not as apparent in the ethidium bromide stained slab gel of Figure 2.

Second-dimensional analysis of DNA from the monomer/dimer/trimer region of the tube gel designated in Figure 4 revealed that the primary separation of DNA was on the basis of size, since most of the DNA migrated along the diagonal (Figure 5). The monomer region was diffuse in that it consisted of a major oval-shaped spot [mononucleosome DNA, ~160 to ~250 base pair(s) (bp)] with a front of staining material (core DNA, ~140 to ~160 bp). The dimer region appeared similar to the monomer region, consisting of a major oval-shaped spot (centered at ~400 bp) with a faster moving front-running fraction which merged into the monomer size DNA region; there was also a tail of larger DNAs (centered at ~460 bp) which overshadowed the major spot of dimer DNA. The presence of so much off-diagonal material (instead of a narrow diagonal line of DNA) suggested that the heterogeneity of nucleosome dimers was greater than that previously indicated by the three dimer subsets in the A_{260} profile of the first-dimension gel. A similar situation was also evident in the trimer region where nucleosomes containing larger trimer DNA fragments migrated ahead of, or coincident with, nucleosomes containing smaller trimer DNA fragments in the first-dimension (nucleoprotein) gel. Thus, although the nucleoproteins had been separated primarily by the nucleosomal DNA size class (i.e., monomer, dimer, trimer, tetramer), the separation of nucleoproteins within a particular size class appeared to be partly determined by other factors. Analysis of the proteins in the second dimension (below) allowed us to draw some general conclusions with regard to the secondary determinants of sea urchin nucleoprotein separation in polyacrylamide gel electrophoresis.

When the constituent proteins of each nucleosomal component were analyzed by electrophoresis in a second-dimension (Triton) acid/urea gel (Figure 6), we found that the intensity of Coomassie blue stained proteins across the gel correlated with the peaks observed in the A_{260} profile of the total nucleoprotein and that, within a given nucleosomal size class,

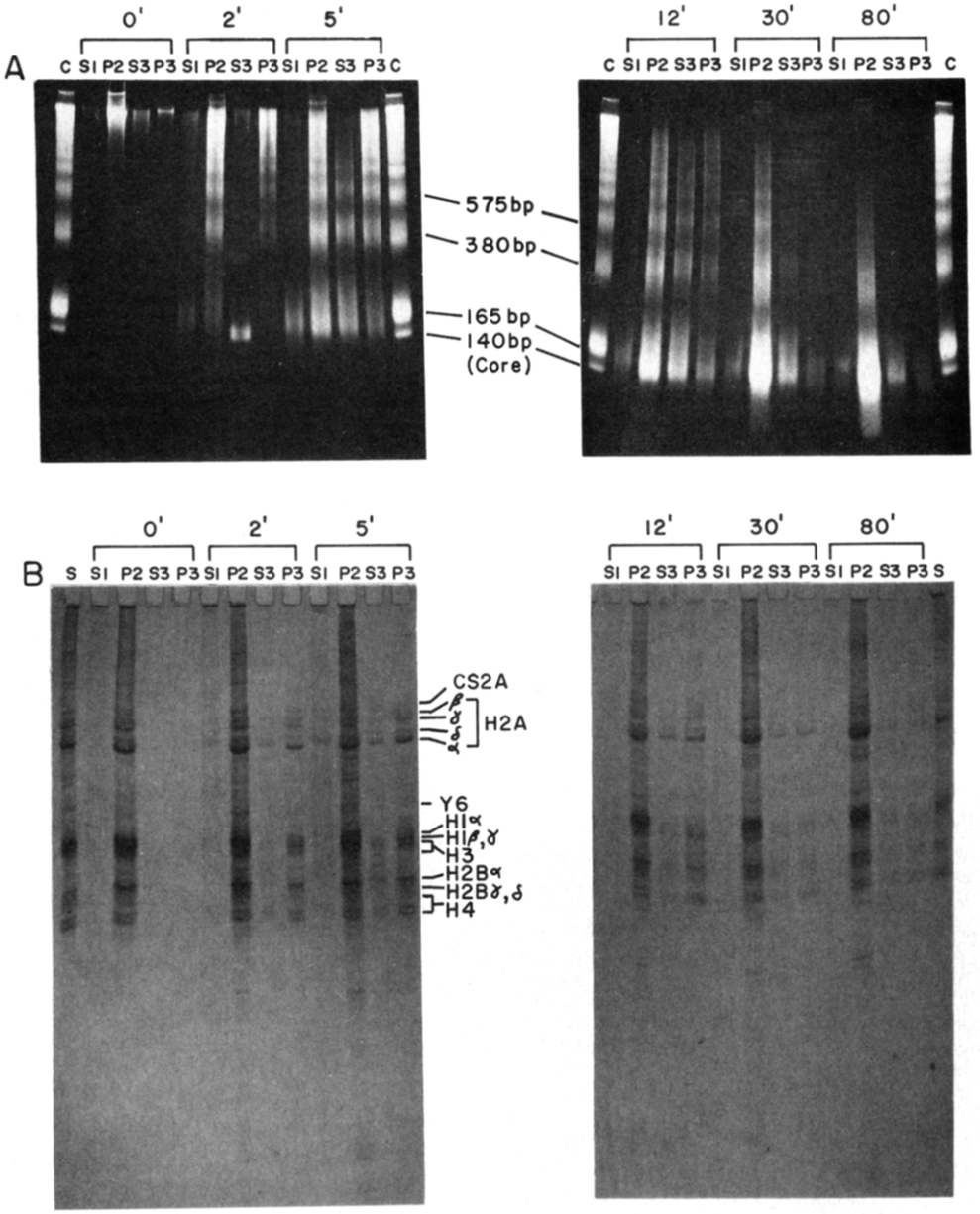

FIGURE 3: Electrophoretic analysis of DNA and protein components in prism-stage nucleoprotein fractions of Figure 2. Prior to DNA and protein analysis, the nucleoprotein fractions were dialyzed vs. a low ionic strength buffer (2 mM Tris and 0.2 mM EDTA, pH 7.75) to remove salt, and the total sample was used for analysis. (A) DNA analysis. Samples containing $\lesssim 1$ A_{260} unit of nucleoprotein were separated on 4% polyacrylamide gels supplemented with 0.1% SDS. A sample of phenol-extracted DNA (C) from a 3-min micrococcal nuclease digest (125 units/mL) of chicken erythrocyte nuclei (25 A_{260} units/mL) was also electrophoresed for comparison. The DNA was stained with ethidium bromide. (B) Protein analysis. Proteins of each fraction were released from DNA by the in situ protamine-release method and separated in (Triton) acid/urea gels (8.5 in. long). Following electrophoresis at 250 V for 10 h (water cooled), the proteins were stained with Coomassie blue. Proteins from an unfractionated sample of prism-stage nucleoproteins were treated in the same way and run as a standard (S).

nucleosomes having H1 or non-histone proteins migrated slower than nucleosomes lacking the proteins. In order to see this more clearly, the H1α band (the darkest staining protein component visualized by Coomassie blue) was scanned horizontally across the oligomer, dimer, and monomer regions and compared to the A_{260} profile of the total nucleoprotein. As seen in Figure 4, the peaks in the scan of the H1α band corresponded closely to nucleoprotein subsets detected by scanning at A_{260}. This comparison demonstrated the similarity of the two profiles and confirmed our previous conclusion that electrophoretic fractionation of this nucleoprotein was able to separate defined, though not necessarily homogeneous, subsets of nucleosomes.

In order to provide a more quantitative estimate of the relative proportions of individual proteins in the nucleosome subsets, the dimer region of the gel (in Figure 6) was cut vertically for scanning into four equal sections [approximating the positions of the four peaks observed in the scan of histone H1α (Figure 4)]. We concentrated on nucleosomes in the dimer region because it provided us with subsets of nucleoproteins that were homogeneous in the number of nucleosome subunits yet migrated with different mobilities. In contrast, the oligomer region consisted of nucleoproteins corresponding to both trimer and tetramer nucleosomes, and the quantity of protein in the monomer region was not sufficient for scanning. Also, we had previously reported that faster migrating monomer nucleosomes from the blastula stage are reduced in their amount of H1 histones compared to slower migrating monomers [cf. Richards & Shaw (1982)].

The scans in Figure 7 showed that one difference in the protein composition of the dimer nucleoproteins was the presence of small quantities of four major non-histone types,

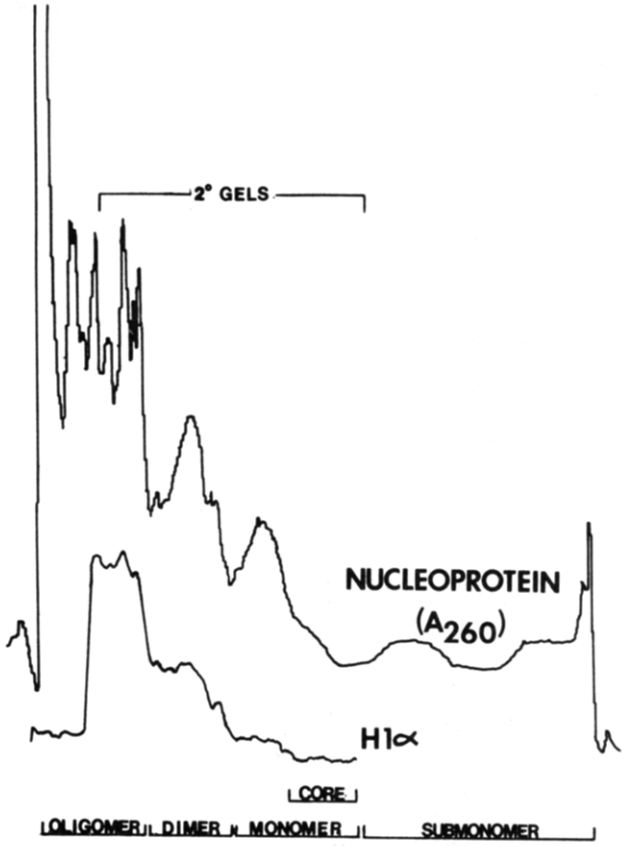

FIGURE 4: Profile of electrophoretically separated nucleoproteins in the blastula-stage 5-min P3 fraction. Nucleoproteins (~5 A_{260} units) were electrophoretically separated in a 4% polyacrylamide tube gel and subsequently scanned at 260 nm. Populations of monomer, dimer, and oligomer nucleosomes were found to be heterogeneous. The portion of the gel designated "2° gels" was analyzed for DNA (Figure 5) and proteins (Figure 6) in the second dimension. Following second-dimension electrophoresis of proteins in a (Triton) acid/urea slab gel, the Coomassie blue stained H1α band was scanned horizontally. The peaks observed in this band closely correspond to the nucleoprotein peaks observed in the A_{260} profile.

designated NHP1, NHP2, NHP3, and NHP4. Whereas all four types appeared to be present in the two slowest migrating dimer subsets (region A and region B), the faster migrating subset (region B) had a reduced amount of NHP1. Moreover, the next subset of dimers (region C) had almost no NHP1 or NHP2 and proportionally more NHP3 and NHP4. The fastest migrating dimer subset (region D) appeared to be deficient in non-histone proteins. Although these non-histone proteins have not been identified, one of them appears to migrate in the position of the Y6 protein identified by Newrock et al. (1978a).

In order to determine any variation in the histone composition of these nucleoprotein subsets, the relative amounts of histones were compared by evaluating the areas under the curves as shown in Figure 7. Since the major histone bands were sufficiently well resolved and intense enough for analysis, the amounts of H2Aα, H2Bα, and H1α therein were normalized to the amount of total histone H4 in each of the dimer subsets. The assumption of a constant value of two H4 molecules per nucleosome provided an internal standard for comparing the relative quantities of these histone variants. The staining intensities of the minor histone components (CS, β, γ, δ) were too weak to be reliable, and we therefore did not attempt to interpret the data for these proteins. Since Coomassie blue binds to varying degrees depending on the histone (Ring & Cole, 1979), differences in protein staining intensity cannot be interpreted in an absolute sense. Nonetheless, the

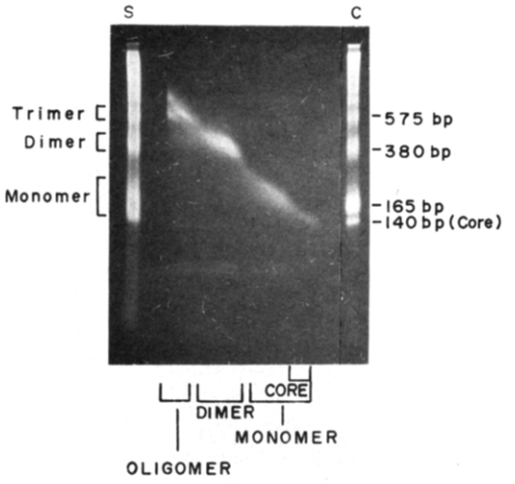

FIGURE 5: Second-dimension DNA analysis of nucleoprotein in the blastula-stage 5-min P3 fraction (see Figure 2). Electrophoresis of nucleoproteins in the first dimension was from left to right; electrophoresis of DNA in a 4% polyacrylamide slab gel supplemented with 0.1% SDS was from top to bottom. First- and second-dimension electrophoresis and the staining of DNA with ethidium bromide were carried out as described under Experimental Procedures. Samples in the side wells show the one-dimensional separation of DNA in the blastula-stage 5-min P3 fraction (S) and DNA from a 3-min micrococcal nuclease digest (125 units/mL; 25 A_{260} units/mL) of chicken erythrocyte nucleic (C) for comparison.

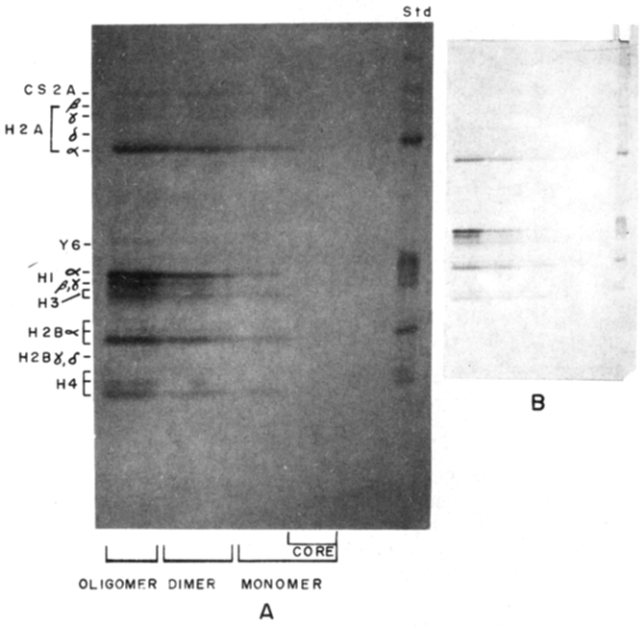

FIGURE 6: Second-dimension protein analysis of nucleoproteins in the blastula-stage 5-min P3 fraction. Electrophoresis of nucleoprotein was from left to right; electrophoresis of protein in a (Triton) acid/urea gel was from top to bottom. Proteins were released from DNA by the in situ protamine-release method and electrophoresed in a 21 × 22 × 0.3 cm^3 slab gel equipped with a water cooling chamber for 20 h at 125 V, followed by staining with Coomassie blue. A sample of proteins released from a whole micrococcal nuclease digest of blastula-stage nuclei by the in situ protamine-release method was electrophoresed as a standard (Std). (A) Histone region only; (B) full-length view of the gel. There are no detectable high molecular weight non-histone proteins.

trends which we report here should be qualitatively significant for the major histones.

The results of this analysis (Table I) indicated that (i) the amount of histone H1α was reduced by approximately one-third in the fastest migrating dimer subset compared to the other three and (ii) the amounts of H2Aα and H2Bα decreased from the slowest to the fastest migrating dimers by

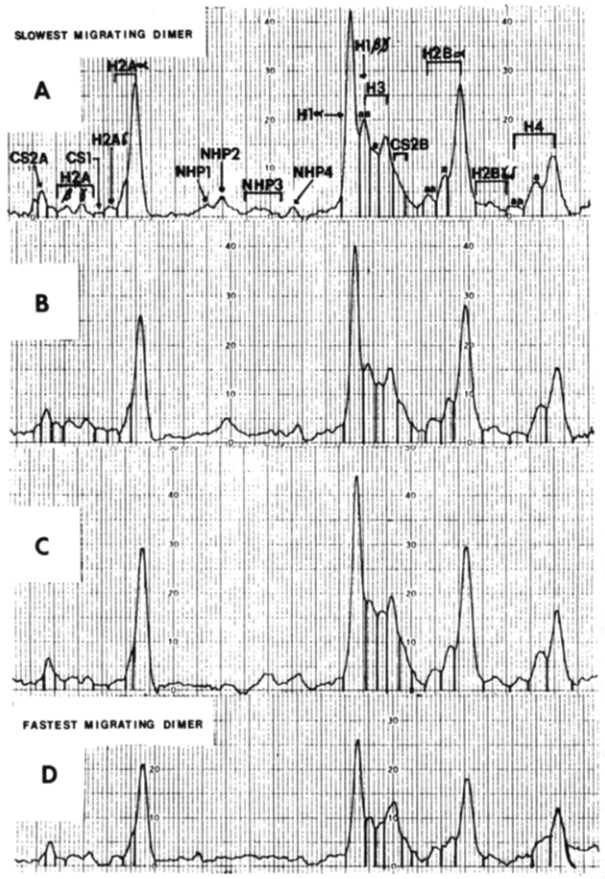

FIGURE 7: Vertical scans of electrophoretically separated proteins from blastula-stage dimer nucleosomes. The dimer region of the second-dimension (Triton) acid/urea gel shown in Figure 6 was divided into four equal sections and scanned vertically. Scan A corresponds to the slowest migrating dimer; scan D corresponds to the fastest migrating dimer. NHP1, -2, -3, and -4 non-histone proteins are asymmetrically distributed across the dimer region. To estimate the relative proportions of variant histone subtypes, the designated areas under the curves were quantified by the method of cut and weigh. The results of this analysis are tabulated in Table I.

Table I: Proportional Amounts of Major Histone Variants in Dimer Nucleoproteins Normalized to Total Histone H4[a]

	region[b]			
histone(s)	A	B	C	D
H1α	1.35	1.22	1.26	0.89
H2Aα	1.04	0.97	0.93	0.95
H2Bα	1.23	1.18	1.04	0.89
H4	1.00	1.00	1.00	1.00

[a] The area in each histone peak was divided by the sum of all the histone peaks including minor histones to yield a percentage of total histones at each of the four points across the dimer region. This percentage value was then normalized to the sum of the total of all histone H4 bands within that dimer subset. Analysis indicated a standard deviation of 5% of the mean for the major histone subtypes, and we estimated that the minimum random error in this analysis was on the order of its standard deviation, or ±5%. A complete analysis of these data is given in Richards (1982). [b] Migration rate increases from region A to region D.

9% and 28%, respectively. We concluded that the values obtained for H1α were reasonable, since histone H1 is associated with spacer DNA and a reduced amount of histone H1α in the fastest migrating dimer would be expected for dimer subsets having truncated DNA fragments (Figure 6). Our results also agreed with the finding in other organisms that the presence or absence of histone H1 influenced the electrophoretic separation of monomer nucleosomes (Varshavsky et al., 1976; Boulikas et al., 1980). From the consistency of these results, we inferred that the trend with regard to histone H2Bα was significant insofar as it suggested a microheterogeneity of core histone composition across the dimer region. The possibility of a decrease in H2Aα was intriguing since it could imply some linked relationship between histones synthesized at similar times in development.

In summary, the electrophoretic migration of specific dimer subsets was at least partly determined by the numbers and amounts of non-histone proteins and histone H1 associated with the nucleosomes. The relatively small amounts of non-histone proteins compared to the histones, however, implied that only a small proportion of the dimers was bound with basic non-histone proteins and that other factors influenced the migration of the remaining dimers which lacked non-histones. Although the observed heterogeneity in electrophoretic mobility might be explained by variations in histone modifications that influence the net nucleosome charge, we have no evidence to that effect. The observed differences in the core histone populations across the dimer region suggested the possibility that this heterogeneous distribution of nucleoproteins might have resulted from the partial separation of conformationally different nucleosomes.

Discussion

Our results indicate that subtle changes in chromatin structure occur through sea urchin early development, as evidenced by a simultaneous decrease in nucleoprotein solubility and digestion to different electrophoretic classes of mononucleosomes. Morula-stage nucleosomes are nearly all soluble in low ionic strength buffers, whereas prism-stage nucleosomes are mostly insoluble (Figure 2). Morula- and blastula-stage chromatins are more readily degraded by micrococcal nuclease to a distinct class of slower migrating mononucleosomes, whereas prism-stage chromatin appears to be digested directly to nucleosomes migrating in the region of cores. Moreover, there is an increasing complexity in the nucleoproteins of the developing sea urchin embryo as development proceeds. At morula stage, the major differences among electrophoretically separated monomer nucleosomes can be accounted for by the presence or absence of histone H1. However, by blastula stage, the discrete bands observed at morula stage are superimposed over an indiscrete background of nucleoproteins, and by prism stage, there are only broad diffuse bands of nucleoprotein in the monomer and dimer regions. Since the average repeat length and the distribution of DNA sizes arising from nuclease digestion remain fairly constant between these stages of development (Keichline & Wassarman, 1977, 1979; Arceci & Gross, 1980; G. Cognetti and B. R. Shaw, unpublished results), this increasing number of nucleoproteins cannot be simply attributed to a greater number of DNA sizes (within a particular nucleosome subunit class) from one stage of development to another.

It has been demonstrated in other organisms that H1-associated mononucleosomes migrate more slowly than H1-depleted nucleosomes (Boulikas et al., 1980; Varshavsky et al., 1976). This is also true in the sea urchin, wherein we demonstrated that blastula-stage H1-containing nucleosomes likewise migrated as discrete bands behind the core (Richards & Shaw, 1982, and this paper). However, in prism stage, there were no discrete nucleoprotein bands migrating behind the core in the monomer region of electrophoretically separated nucleoproteins (Figure 2). Protein analysis (Figure 3), however, revealed that β, γ, and δ (late) subtypes of histone H1 were present predominantly in the prism-stage P2 fractions where monomer spreading was most pronounced. These H1 histones

were greatly diminished in the S1, S3, and P3 fractions which had most of the nucleoproteins migrating in the core region on nucleoprotein gels. Thus, although there were no discrete monomer bands at prism stage, the presence of late H1 variants correlated with a decrease in both the electrophoretic mobility and solubility of prism-stage nucleoprotein. We therefore attributed some of the observed microheterogeneity in the prism-stage P2 fractions to the presence of the three late major subtypes of histone H1.

Analysis of a single fraction of nucleoprotein from blastula-stage embryos gave support to such an explanation and provided us with a better perspective of nucleosome microheterogeneity in the developing sea urchin embryo. In a 0.1 M NaCl insoluble fraction of a 5-min micrococcal nuclease digest, we found four subsets of dimer nucleosomes that differed in their composition of H1 and non-histone proteins, with the slower migrating subsets enriched in these proteins. We also found proportionately more early stage H2Bα histones in slower migrating dimers than in faster migrating dimers, and smaller variations in the relative proportions of the H2Aα variant across the dimer region. In view of our observation of an increasing complexity of nucleoprotein through development, these results are consistent with the idea that electrophoretic migration of these dimer nucleoproteins is in part determined by the core histone composition of the nucleosomes. Conformational differences in nucleosomes having different histone variants might give rise to electrophoretic heterogeneity.

Although we have no direct evidence that histone variants would alter nucleosome conformation and consequently the electrophoretic mobility, the work of Simpson (1981) and Shaw and Richards (unpublished results) shows that nucleosome cores from blastula- and pluteus-stage embryos (containing different histone variant populations) exhibit slightly different sedimentation properties at low ionic strength. Because our electrophoretic separation of nucleosomes was carried out here under conditions of low ionic strength, it seems likely that the molecular sieving properties of a polyacrylamide gel might be sensitive to conformational differences induced by coordinate changes in histone core and H1 variant conformations, particularly if specific H1 variants are predisposed to interact with their homologous core histone variants. Clearly these differences will need to be more rigorously defined for future studies of nucleosome structure–function relationships in the developing sea urchin embryo.

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