Structural differences in the chromatin from compartmentalized cells of the sea urchin embryo: differential nuclease accessibility of micromere chromatin

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Received 13 July 1981

ABSTRACT

The chromatin structure of three cell types isolated from the 16-cell stage sea urchin embryo has been probed with micrococcal nuclease. In micromeres, the four small cells at the vegetal pole, the chromatin is found to be considerably more resistant to degradation by micrococcal nuclease than chromatin in the larger mesomere and macromere cells which undergo more cellular divisions and are committed to different developmental fates. The micromeres show an order of magnitude decrease in the initial digestion rate and a limit digest value which is one third that of the larger blastomeres; both observations are suggestive of the formation of a more condensed chromatin structure during the process of commitment, or as the rate of cell division decreases. The decreased sensitivity to nuclease for micromeres is similar to results reported for sperm and larval stages of development.

INTRODUCTION

The echinoids are receiving renewed attention in molecular embryology for studies of gene and chromatin structure during early development. There are reproducible, stage-dependent changes in the H1, H2A and H2B histones of sea urchin embryos¹⁻⁶ and corresponding switches in histone messenger RNAs during development [as reviewed by Kedes⁷]. As with other eukaryotic systems, sea urchin chromatin exhibits a nucleosomal repeat⁸⁻¹² and core particle structure¹³. During development, the repeat length of the nucleosome¹² and the core particle composition¹³ changes systematically and in parallel with the histone composition of the chromatin. Likewise, as development proceeds, a systematic and decreased availability of chromatin to attack by nuclease is observed^{9,12}, and a notable resistance to nuclease offered by the unfertilized egg recently has been reported¹⁴.

It therefore seemed warranted to determine whether there is a correlation between nuclease resistance in chromatin, and the state of

differentiation of the embryo. We examined the chromatin structure of individual cell types in the 16-cell embryo, where the beginning steps in the differentiation process can be observed. The cells at this point are no longer identical in size and have lost the capability to form normal embryos if separated. Three classes of cells can be distinguished: the mesomeres (eight in number) at the animal pole; the micromeres (four small cells at the vegetal pole) and the macromeres (four large cells in between the mesomeres and micromeres)¹⁵. Nuclei from each of the three blastomere types were treated with micrococcal nuclease in order to probe for structural differences in the chromatin which might correlate with the beginning of differentiation.

MATERIALS AND METHODS

Fertilization and Culture of Embryos. Adults of Arbacia punctulata and Strongylocentrotus purpuratus were supplied by the Duke Marine Biological Laboratory of Beaufort, NC and Pacific BioMarine, Venice, CA, respectively; Arbacia lixula were collected from the Gulf of Palermo, Sicily. Eggs were collected by spontaneous shedding of scissor-opened females. Samples from each batch of eggs were test fertilized and only those which showed $\geq 90\%$ of fertilization were employed. Fertilization took place with a minimum excess of sperm and in the presence of 1mM aminotriazol, used to prevent the hardening of the fertilization envelope¹⁶. Fertilized eggs were washed several times with artificial sea water (Instant Ocean) to remove excess sperm; polyspermy was never observed. The embryos were monitored for normal cleavage and only cultures showing good synchrony and normal development were grown to the 16-cell stage under gentle stirring at 18°C and at a concentration ≤ 5000 embryos/ml in artificial sea water containing penicillin and streptomycin. [³H]-Thymidine was added soon after fertilization at a concentration of 0.25 μ Ci/ml and a specific activity of 40-60 Ci/mmol.

Removal of Fertilization Envelope and Cell Separation. Fertilization envelopes were removed by forcing the suspension of embryos two or three times through a 50 μ m nylon screen followed by several low speed centrifugations. Dissociation took place in CMFSW (calcium-magnesium-free sea water) and 2mM EDTA; macromeres, mesomeres, and micromeres were isolated on 5-15% Ficoll gradients as described by Hynes and Gross¹⁷.

Nuclei Isolation and Digestion. Purified nuclei were isolated in the presence of LiCl, which inhibits the endogenous nuclease^{13,14}. The

macromere, mesomere and micromere cells were harvested by low speed centrifugation, washed twice in IG buffer (0.1 M Na phosphate, 0.1 M LiCl, 0.5 mM phenylmethylsulfonyl fluoride, pH 6.0) supplemented with 24 mM Na₂EDTA, pH 6.0, and pelleted at 4,000 x g in a Sorvall HB-4 rotor for 10 min. This was followed by a wash in IG buffer without Na₂EDTA, and further centrifugation. The pellet was homogenized in IG buffer using a Dounce homogenizer, closely monitoring the extent of cell lysis through the microscope. Triton X-100 was then added (to a final concentration of 0.2%) to the broken cell suspension and the nuclei allowed to incubate for a further 5 min at 0°. These were pelleted at 2600 x g for 10 min and carefully resuspended and washed two times in IG buffer. For nuclease digestion, the nuclei were washed several times in KPS buffer (.085 M KCl, 0.3 M sucrose, 5 mM Pipes-HCl, pH 7.0) and resuspended in KPS buffer. Volumes were adjusted in order to have approximately the same nuclease/nuclei ratio in each of the macromere, mesomere, and micromere suspensions. The digestion with 20 units/ml micrococcal nuclease (Worthington) and 1 mM Ca⁺⁺ was carried out at 37°C on intact nuclei suspensions from macromeres, mesomeres and micromeres at a concentration of 14-40 A₂₆₀/ml. Samples were withdrawn at various times and the digestion stopped by the addition of an equal volume of 10 mM EDTA. Controls with Ca⁺⁺ were incubated under the same conditions for the same length of time as the longest time points, with an equal volume of distilled water replacing the nuclease.

DNA Extraction and Electrophoresis. Part of each time-digest sample was treated with RNase A (10 µg/ml) and Proteinase K in the presence of SDS and extracted with phenol and chloroform:isoamyl alcohol according to Shaw *et al.*¹⁸. After ethanol precipitation the DNA was dissolved in 1/10 E buffer for electrophoresis¹⁹.

Perchloric Acid Precipitation and Counting. Part of each time-digest sample was treated at 0°C with 10% perchloric acid, 1M NaCl in order to have a final concentration of 5% PCA, 0.5M NaCl. Samples were centrifuged at 15,000 rpm for 10 minutes. The supernatants were carefully and completely withdrawn with a Pasteur pipet. Pellets or supernatants were individually treated with Protosol (New England Nuclear) and after solubilization mixed with toluene-PP0-POPOP scintillation cocktail and counted. Percent digestion was calculated as supernatant ÷ (supernatant + pellet).

RESULTS

The three cells from highly synchronous cultures of 16-cell embryos were separated using Ficoll gradients and their nuclei isolated and digested with micrococcal nuclease. Figures 1a and 1b show the nuclease digestion pattern of DNA isolated from each of the three nuclear suspensions. After 4 minutes of incubation with nuclease (Fig 1a), the chromatin from macromeres and mesomeres is extensively digested, showing a monomer, dimer, and multimer subunit pattern characteristic of nucleosomes. By contrast, the chromatin from micromeres, incubated under similar conditions, shows only slight digestion after 4 minutes. After 16 minutes the digestion proceeds further for macromere and mesomere chromatin while only a slight amount of the monomer nucleosome appears for micromeres. A complete nucleosomal pattern for the micromeres appears only after 64 minutes, at which point the macromeres and mesomeres have been extensively digested, as seen by the small amount of multimer bands and the large amount of submonomers (invisible for micromeres) present in the mesomere and macromere digests. A more dramatic view is seen in Fig. 1b which shows the zero and very long digestion times. Even after 9 hours of digestion the monomer-dimer-trimer-tetramer pattern is visible for DNA of the micromere chromatin, whereas macromere and mesomere DNA is completely digested. The close correspondence in intensities of the nuclease patterns on the fluorographed gel (Fig. 1 bottom) and that stained with ethidium bromide (Fig. 1 top) are evidence that the preparations were not contaminated with significant amounts of unlabeled DNA from entrapped sperm heads or non-nucleosomal mitochondrial DNA. Similar results with nuclei have been observed in every set of experiments (five) carried out to date. Preliminary results on the digestion of chromatin isolated from the three cell types show a similar, but less dramatic trend, wherein the micromere chromatin digests at a rate 4-times slower than that of macromeres (data not shown).

The data suggest that chromatin of micromeres is more resistant to nuclease than chromatin of mesomere and macromere cells. Because endogenous nucleases have been reported in chromatin preparations of echinoids^{20,8} and because their levels of concentration vary with development (being minimal in cells which divide slowly or not at all²¹), it was important to eliminate selective activation of an endogenous nuclease as a cause of the enhanced degradation of DNA in the macromere

Fig. 1 Nuclei were isolated from macromere, mesomere and micromere cell populations (A. punctulata) grown continuously in the presence of ^3H -thymidine; they were digested (at $37\text{ A}_{260}/\text{ml}$ for macromeres, $26\text{ A}_{260}/\text{ml}$ for mesomeres, and $14\text{ A}_{260}/\text{ml}$ for micromeres) with 20 units/ml micrococcal nuclease and 1 mM CaCl_2 in KPS buffer. The digestion was stopped at specified times with 5 mM EDTA and the DNA was extracted with Proteinase K, phenol and chloroform/isoamyl alcohol, followed by precipitation with ethanol, treatment with ribonuclease A, and re-precipitation; DNA was electrophoresed on a 3% polyacrylamide slab gel. PM2-Hae III fragments were used as standards. Although the ratio of nuclease to DNA was in effect $2\frac{1}{2}$ times greater for the micromeres as for the macromeres, the micromere digestion proceeded considerably slower than the mesomeres and macromeres. Gels at the top were stained with ethidium bromide. Gels at the bottom were fluorographed.

and mesomere cells. Thus the LiCl-phosphate buffer at low pH was selected for nuclear isolation, since it is an effective inhibitor of endogenous nuclease activity¹⁴.

To establish that the levels of endogenous nuclease activity were indeed low, we analyzed the digestion kinetics for DNA in nuclei isolated from macromeres, mesomeres, and micromeres that were incubated in the presence and absence of micrococcal nuclease. Growing embryos were labeled with ³H-TdR for the first four divisions and the amount of acid soluble ³H-DNA was monitored as a function of digestion time for each cell type (Fig. 2). The amount of acid soluble DNA released in the controls after four hours incubation in the presence of 1 mM CaCl₂, but without micrococcal nuclease, shows no variation from the zero-time value for each of the cell types, ruling out the possibility that the different digestion kinetics arise from different levels of endogenous nuclease activity in the nuclei (Fig. 2 and Table I). As seen in Fig. 2, nuclei

Fig. 2 Digestion kinetics of DNA in nuclei isolated from macromere, mesomere and micromere cell populations. *A. punctulata* embryos were labeled from fertilization to harvest with 1 μ Ci/ml ³H-thymidine; isolated nuclei in KPS buffer were digested at a concentration of 22 + 2 A₂₆₀/ml with 20 units/ml micrococcal nuclease and 1 mM CaCl₂. Control samples were incubated in the presence of calcium and in the absence of micrococcal nuclease.

TABLE I
Kinetics of Digestion of Nuclei from *A. punctulata* Blastomeres

	MACROMERES (20 A_{260}/ml) [†] (5.4×10^5 cpm/ml) $A_{260}:A_{280} = 1.18$ $A_{260}:A_{320} = 4.2$	MESOMERES (24 A_{260}/ml) [†] (6.2×10^5 cpm/ml) $A_{260}:A_{280} = 1.15$ $A_{260}:A_{320} = 3.6$	MICROMERES (21 A_{260}/ml) [†] (4.0×10^5 cpm/ml) $A_{260}:A_{280} = 1.18$ $A_{260}:A_{320} = 2.9$
Digestion Time	% Digestion*	% Digestion*	% Digestion*
0	0.9	0.9	1.2
1'	2.0	1.1	1.2
4'	9.6	3.6	1.6
16'	24.8	22.6	4.1
64'	58.8	61.7	14.0
4 hr	73.8	59.3	21.9
4 hr (no nuclease) control °	1.1	1.3	1.0

[†] For absorbance determinations, samples were dispersed in 2% SDS

* For determination of the percent of digestion, 50 λ aliquots were treated with perchloric acid and counted (see text)

° In the presence of 1 mM $CaCl_2$

from the three cell types are digested with different kinetics. In the presence of micrococcal nuclease, each cell type shows a typical digestion curve, with the micromere nuclei digestion proceeding much (12-times) slower and to a lower limit. Small differences between the macromere and mesomere kinetics may be due to the uncertainties of the experimental method. The lower value for the limit digest and the 12-fold difference in the initial rate of DNA digestion of the micromeres, together with the absence of endogenous nuclease activity in all three cell types, confirmed that the chromatin from micromeres shows a marked resistance to nuclease digestion.

In Table II, the final fraction of DNA digested by micrococcal nuclease for the individual 16-cell stage blastomeres from two species of sea urchins is compared with the limit digest fraction observed for cells at later developmental stages. Whereas the percentage of nuclease accessible DNA in mesomeres and macromeres (60-70%) is similar to that (70-80%) of early embryos at the morula and blastula stages, the levels of digestible DNA drops by two-thirds in the micromere cells (22-27%), closely approximating that (20-30%) observed for sperm and larval stages

Table II

STAGE	FINAL FRACTION DIGESTED (% ACID SOLUBLE DNA)	SPECIES
Macromeres	72% a 69% a	<i>A. punctulata</i> <i>A. lixula</i>
Mesomeres	62% a 69% a	<i>A. punctulata</i> <i>A. lixula</i>
Micromeres	22% a 28% a	<i>A. punctulata</i> <i>A. lixula</i>
Morula	70-80% b	<i>A. punctulata</i>
Hatching blastula	70-80% b	<i>A. punctulata</i>
Blastula	82% c	<i>L. pictus</i>
Mesenchyme blastula	55-65% b	<i>A. punctulata</i>
Gastrula	60% c 45-55% b	<i>L. pictus</i> <i>A. punctulata</i>
Pluteus	47% c 40-45% b	<i>L. pictus</i> <i>A. punctulata</i>
11-Day larvae	34% c	<i>L. pictus</i>
Sperm	~30% d 20% c	<i>P. lividus</i> <i>L. pictus</i>

a this work

b From Ref. 8

c From Ref. 12

d From Ref. 23

of development.

DISCUSSION

One of the interesting features in the development of the sea urchin embryo is the segregation at the fourth cleavage of a small group of blastomeres called micromeres, which derive from the unequal division of the four vegetal blastomeres. This segregation marks the beginning of differentiation, such that in the 16-cell-stage embryo there are three cell types: macromeres, mesomeres, and micromeres. The micromeres are committed to form the primary mesenchyme from which the skeleton eventually arises. The mesomeres and macromeres form the ciliated

epithelium, the archenteron, and the structures derived from them. Thus, these cell types are already committed at the 16-cell-stage. Cell divisions are highly synchronous until the fourth cleavage, after which a gradient-like distribution of cell division sets up along the vegetal-animal axis, ultimately resulting in the segregation of the micromeres.²² Therefore, from the 16-cell stage on, the synchrony of cell division is lost²².

We report that the chromatin in nuclei of the micromere cells is more resistant to digestion with micrococcal nuclease than that of the mesomere and micromere cells. This resistance to micrococcal nuclease action has been observed in cells from the 16-cell stage of three species of sea urchins: A. punctulata, A. lixula, and S. purpuratus (data not shown). To a lesser degree it is also apparent in digestions of chromatin (data not shown). Our observations could be interpreted as indicating that cells which undergo the first step in differentiation exhibit a reduced accessibility to nuclease.

Among several species of urchins the chromatin becomes relatively more inaccessible to digestion as development proceeds^{8,11,12,23}, as seen in Table II; corresponding to this resistance to nuclease is an increase in the observed nucleosomal repeat length¹². The resistance of the micromere cells to nuclease which we have observed approximates the limit digest values which are observed for the older, highly differentiated larvae and sperm cells (Table II). Whereas less than one-third of the DNA in micromeres is solubilized by micrococcal nuclease (compared to 34% in 11-day larvae and 20% in sperm), the ~70% limit digest for the mesomeres and macromeres is similar to that observed for morula and blastula stage cells. Although a number of factors may contribute to lower the limit digest value, including a decrease in the accessibility of nucleosomal DNA and a differential precipitation due to the nature of proteins associated with the DNA, the fact that a difference was observed among the three cell types is itself indicative of dramatic changes in the chromatin structure. In this connection it is interesting to note that the micromeres are committed to only 1-4 more cell divisions, and in fact nearly stop dividing (relative to other cells) at an early stage of development²².

We have shown that the higher resistance to the nuclease offered by the micromere chromatin is not an artifact of endogenous nuclease activity. Since a lower nuclease/nucleic acid ratio in the micromeres

could result in an apparent resistance to nuclease, the nuclease/nucleic acid ratio in the micromere digestion mixture was maintained at a level never less than that of macromeres and mesomeres. Furthermore, analysis of the amount of ethidium bromide staining material in the zero-time samples (Fig. 1), the absorbance at 260 nm, and the amount of ^3H -DNA in each digestion mixture (Table I), indicates that the slight differences in DNA concentration among nuclei of the three cell types cannot account for the 12-fold decrease in rate of digestion of the micromere cells. Also, the relatively constant specific activity (cpm/ A_{260} ratio) among the three blastomere types indicates that the micromere nuclei population was not contaminated by extra nucleic acids arising from RNA or entrapped sperm heads, neither of which are radioactive. Higher amounts of RNA in the micromere nuclei, which would have also reduced the number of molecules of nuclease available for the DNA, are ruled out because the total amount of RNA has been shown to be less in the micromere than in the other two cell-types^{24,25}.

It is not possible from the data presented to decide whether the observed variations in digestion kinetics are due strictly to differences in chromatin structure or to factors that might influence the overall activity of the nuclease. However, it should be noted that (i) the nuclei from the three cell types were isolated under the same conditions, simultaneously, and with the same reagents; in the digestion they appeared intact and not broken, sticky or clumped. (ii) Whereas a reduced permeability to the nuclease offered by the nuclear membrane of micromere nuclei would produce a diminished rate of digestion, it alone could not account for the low value observed for the limit digest. Furthermore, when chromatin preparations were digested, the micromeres continued to exhibit a reduced digestion rate. Digestion of chromatin itself, rather than nuclei, would avoid problems with membrane permeability but is not necessarily definitive, because chromatin preparations suitable for use in this type of experiment (neither sticky nor clumpy) are likely to have lost the integrity of their superstructure as well as some of their nonhistone proteins, which presumably are responsible for the different digestibility of chromatins. The possibility of a differential permeability to the nuclease by the nuclei of one cell type is also not very likely, due to the presence of nucleopores²⁶. (iii) Differential retention of LiCl or other soluble nuclease inhibitors by the micromere nuclei could also affect the

kinetics. [LiCl inhibits micrococcal nuclease (unpublished observation, Shaw and Cognetti) as well as endogenous nuclease.] To minimize such inhibition, the nuclei from macromeres, mesomeres, and micromeres were extensively rinsed after their isolation and before the digestion. We cannot completely rule out the possibility of a differential retention of a soluble inhibitor by the micromere nuclei, but such a finding might be of equal interest to the finding of a differential digestibility of the chromatin. Experiments to test this are in progress. (iv) The presence of proteases would affect the digestibility of the chromatin; a higher level of protease in macromere and mesomere nuclei might destroy part of the chromatin proteins thereby making the DNA more accessible to nuclease or, conversely, a higher amount of proteases in micromeres might destroy part of the nuclease. However, acid-urea electrophoresis of nuclear proteins from macromere, mesomere, and micromere cells (data not shown) did not reveal any proteolytic degradation of nuclear proteins. We therefore have good reason to believe that the nuclear and chromatin structure of micromeres somehow differs from that found in mesomere and macromere cells of the 16-cell embryo.

The resistance of the micromeres to micrococcal nuclease degradation is interesting in view of the absence of detectable quantities of high complexity nuclear RNA in the micromere cells, though such RNA exists in other cells of the fourth-cleavage embryo and at later stages²⁵. Taken together, both observations imply that there may be a general tightening or compaction of the chromatin structure, which prevents access to the DNA (by either the nuclease or the enzyme complex which carries out transcription) and affects gene expression within major domains of the genome. The lower rate of division for the micromeres after the fourth division²² may likewise result from an increased condensation of the chromatin structure in the micromeres.

These results suggest that special regulators which determine the micromere cell lineage are present in the chromatin of the micromeres. Attempts to ascertain significant differences between the protein synthesis patterns among the three blastomere types failed to uncover qualitative differences within the subset of whole embryo proteins having isoelectric points between pI 4.5-7.2 and molecular weights between 10,000-130,000²⁷. However, detectable differences in the relative ratios of the two H1 histones synthesized in the three cell types of the 16-cell stage embryo²⁸, as well as differences in the ratio of histone to nonhistone

synthesis²⁹, have been observed. Variations in the relative ratios of the two H1 histones may be noteworthy in light of the recent correlation of H1⁰ with nuclear quiescence^{30,31}.

Induction of a unique pattern of micromere nuclear RNA synthesis may be a direct result of the state of chromatin compaction. A correlation between the nuclease resistance of chromatin and the state of differentiation, and/or the growth rate of the embryo, is suggested by our data. These results have implications for the possibility of a diverging chromatin composition and structure in very early development at the time when cell determination is in progress.

ACKNOWLEDGEMENTS

This work was supported by Grant GM-23681 to B.R.S. from the U.S. Public Health Service, Institute of General Medical Sciences. B.R.S. is a scholar of the Camille and Henry Dreyfus Foundation. We thank Drs. S. Chambers, G. Giudice, D. McClay, P. Modrich and Mr. R. Richards for their helpful comments.

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