Journal of Cellular Biochemistry 59:161–167 (1995)

Sea Urchin Zygote Chromatin Exhibit an Unfolded Nucleosomal Array During the First S Phase

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Abstract To investigate changes in chromatin organization associated with DNA replication during the first stages of development of the sea urchin *Tetrapygus niger*, we compared micrococcal nuclease (MNase) digestion patterns of chromatin from zygotes harvested during the first S phase and from unfertilized eggs. We observed that the majority of DNA fragments derived from MNase digested zygote nuclei were similar to or smaller than a mononucleosome, while those derived from unfertilized egg nuclei were larger (1,500 to 410 bp). This result indicates that in zygotes, where active DNA replication is occurring, the major chromatin fraction is represented as unfolded nucleosomes. In contrast, in unfertilized eggs chromatin appears to be organized into polynucleosomes. To determine if the unfolded structure of nucleosomes observed during S phase is related to the level of poly (ADP-ribosylation) of cleavage stage (CS) histone variants, zygotes were treated with 20 mM 3-Amino Benzamide (3 ABA) *during the interval* between 3 and 30 min post-insemination (p.i.). This treatment with 3 ABA decreases the poly (ADP-ribosylation) of CS histone variants and inhibits the first S phase in zygotes [Imschenetzky et al. (1991): J Cell Biochem 46:234–241; Imschenetzky et al. (1993): J Cell Biochem 51:198–205]. When the MNase digested patterns of chromatin from these 3 ABA treated and control zygotes were compared, we found that the unfolded structure of the nucleosomes remains unaltered by the inhibition of the poly(ADP-ribose) synthetase with 3 ABA. This result indicates that the unfolded nucleosomal structure, particular to the chromatin of S phase zygotes, is not contemporaneous to DNA replication and is independent of the normal level of poly(ADP-ribosylation) of CS histone variants.

Key words: micrococcal digestion, chromatin, DNA replication, sea urchins, early development

In most cells the events related to genome replication and cell divisions are paralleled by those related to cell growth. Experimental models that allow exploration of these two events independently are not frequent in vivo. One of the most attractive of such models is the cleavage stages of sea urchins, in which the two sets of events, those associated with DNA replication and cell division and those related to cell growth, are naturally unlinked. In contrast to most cells, cleavage divisions in embryos are characterized by very fast cell cycles with a predominant S phase, an abbreviated G1, and very short G2/M intervals [Ito et al., 1981]. As shown previously, the first S phase is completed within 50 min, while the first cell division occurs 90 min after zygote insemination and is overlapped with the second S phase [Hinegardner et al., 1964]. This extremely high rate of DNA replication, unique to cleavage cells, has been associated with the synchronous activation of the majority of the potential replication origins that occur at the beginning of S phase in early zygotes [Diffley and Stillman, 1990].

In sea urchins the first embryonic cell cycle exhibits a short period of male pronucleus remodelling which precedes the first round of DNA replication [Imschenetzky et al., 1991a]. In monospermic conditions, this period is characterized by the loss of the five sperm specific histones. Consequently, the major basic proteins that are forming zygotic chromatin at the onset of the first replication wave are identical to those found in unfertilized eggs [Imschenetzky et al., 1980, 1995]. It has being previously shown that the chromatin in sea urchin eggs and early cleavage embryos is organized by cleavage stage (CS) histone variants which are normally poly-(ADP-ribosylated). This post-translational modification changes in a cell cycle dependent manner and is related to the normal progress of S phase [Imschenetzky et al., 1991b]. The CS variants differ from typical histones found in most

Received January 19, 1995; accepted February 10, 1995.

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cells and this is reflected by a chromatin organization characteristic of unfertilized eggs and cleavage stage embryos [Shaw et al., 1981; Imschenetzky et al., 1989, 1993a]. As reported previously, the protein oligomer formed by CS histone variants is bound to approximately 126 bp of DNA instead of the 146 bp of DNA that are surrounding the well-defined histone core of nucleosomes found in most cells [Imschenetzky et al., 1989]. Due to these features of cleavage cells, it may be expected that the eventual chromatin structural changes strictly related to DNA replication will be detectable and amplified in these cells.

To study the structural organization of S phase chromatin we have isolated nucleoprotein particles derived from MNase digestion of the nucleus from sea urchin zygotes harvested during the first S phase (40 min p.i.). Then we compared these nucleoprotein particles with those obtained from unfertilized eggs and analyzed the DNA fragments contained in both types. Our results indicate that during S phase normal zygotes are characterized by a chromatin arrangement of sub-nucleosomal or open nucleosomal arrays with a minor proportion of whole nucleosomes.

In addition we determined whether this unfolded chromatin organization is dependent on the progress of S phase by comparing the MNase digestion products derived from nuclei of normal zygotes with those of zygotes treated with 3 ABA (20 mM) which inhibited the first DNA replication wave. The comparison of the nucleoprotein particles, as well as of the DNA fragments derived from these particles, is reported.

MATERIALS AND METHODS

Gametes and Zygotes

Sea urchins *Tetrapygus niger* were collected from the bay of Concepción and maintained at room temperature in an aquarium containing natural sea water under constant aeration. Unfertilized egg and sperm were obtained as described previously [Imschenetzky et al., 1986, 1989]. Egg insemination was performed and the zygotes were cultured for 40 min at room temperature in normal sea water, or alternatively, in sea water containing 20 mM of 3 aminobenzamide (3 ABA). Under our experimental conditions the first S phase occurs in the zygotes between 30 and 50 min p.i. [Imschenetzky et al., 1991b, 1993a].

Nuclei Isolation and Digestion With Nucleases

Unfertilized egg nuclei were isolated by an aqueous ethanol/Triton X-100 procedure according to Poccia et al. [1981]. Micrococcal nuclease (MNase) digestion was performed by the procedure described by Savic et al. [1981], modified as described previously [Imschenetzky et al., 1989]. In a typical experiment, the isolated nuclei were digested for 10 min at 37°C with 70 U of MNase/ml. The reaction was stopped by addition of a cold buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. The nuclear debris were removed by low speed centrifugation and the soluble nucleoprotein particles obtained were analyzed by electrophoresis in 1% agarose gels [Imschenetzky et al., 1989, 1993b].

Electrophoresis of Nucleoprotein Particles

Electrophoresis of nucleoprotein particles was performed using horizontal 1% agarose gels as described by Weintraub [1984]. For visualization of DNA in the nucleoprotein samples, the gels were stained with ethidium bromide at 1 μg/ml, transilluminated by short wave UV light, and photographed through a Wratten 23 A filter onto Polaroid Type 665 film. Lamda DNA digested with restriction nucleases Hind III and Eco R I was used as standard for electrophoretic migration.

Purification of DNA Fragments and Electrophoresis of DNA

Isolation of DNA fragments was performed according to the procedure described previously by Savic et al. [1981].

Electrophoretic analysis of DNA fragments derived from nuclease digestion was carried out in horizontal 1.9% agarose gels containing 0.1% SDS as described by Weintraub [1984]. ϕ X174 DNA fragments digested with restriction nucleases Hae III and Eco RI were used as standards for electrophoretic migration.

Alternatively, low molecular weight DNA fragments derived from micrococcal nuclease digestion were subjected to electrophoretic analysis in 20% (p/v) polyacrylamide gels as described by Sambrook et al. [1989].

To compare more precisely the patterns of DNA fragments derived from nuclease digestion, after each electrophoretic fractionation the gels were photographed and the negatives scanned in a microdensitometer (Joyce Loebl, model MK III CS).

RESULTS

Nucleoprotein Particles Derived From MNase Digestion

To isolate and analyze chromatin nucleoprotein particles, these were obtained from nuclei of unfertilized eggs or from zygotes and were then digested with MNase for 10 min. The generated digestion products were collected and analyzed by electrophoresis in 1% agarose gels. As shown in Figure 1A–C, a broad and uniform distribution of discrete nucleoprotein particles were obtained from unfertilized eggs, as well as from normal and 3 ABA treated zygotes. These nucleoprotein particles were found to be located in a region of the gel corresponding to DNA fragments of 3,400 to 1,900 bp and 2,500 to 1,900 bp, respectively. Zygotes treated with 3 ABA show one major particle migrating in the same position as does the nucleoprotein particle from normal zygotes or unfertilized eggs (2,500 to 1,900 bp). In addition a minor particle located between 1,500 and 1,300 bp of DNA was observed. These results indicate that chromatin in both eggs and zygotes is normally organized in nucleoprotein particles exhibiting very similar electrophoretic migrations. The nucleoprotein particle structure in zygotes was altered by the treatment with 3 ABA, because a smaller and less defined nucleoprotein particle appeared as a minor component.

MNase Digestion Patterns of Isolated Nuclei

Nuclei isolated from unfertilized eggs or from zygotes were digested for 10 min with MNase and the generated DNA fragments were isolated and analyzed electrophoretically in agarose gels. We found significant differences between the DNA fragment pattern of unfertilized eggs and that of zygotes (Fig. 2A–C). A periodic distribution of well-defined DNA fragments was obtained from unfertilized eggs, with sizes ranging between 1,550 and 410 bp of DNA and preferential DNA cutting sites at 1,550, 980, and 760 bp (Fig. 2A). In contrast the DNA fragments detected after 10 min digestion of zygote nuclei were found to migrate to the bottom of the gel as broad unresolved regions of low molecular weight DNA (Fig. 2B,C).

When a milder digestion procedure was used (3 instead of 10 min of MNase digestion) a complex pattern of migration was obtained both from control and from zygotes treated with 3 ABA. A background smear was impossible to avoid (Fig. 3). As it becomes more clear in the densitograms of these gels, the sizes of the majority of these very heterogeneous DNA fragments are distributed between 130 and 40 bp of DNA, although two additional preferential cutting sites were found, one at 190 bp and the other at 281 bp of DNA. As shown in Figure 3, 3 ABA treatment did not have a significant effect in the composition of this complex DNA fragment pattern in zygotes.

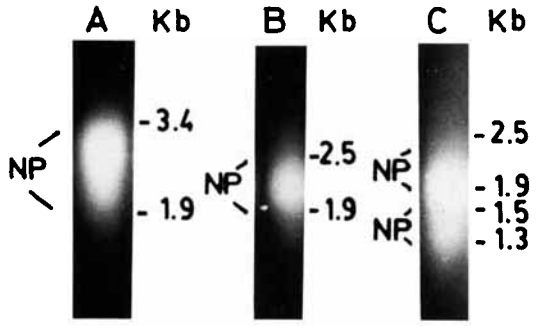

Fig. 1. Nuclei were isolated from unfertilized eggs and zygotes and digested with MNase for 10 min. The MNase digestion products were separated by electrophoresis in 1.5% (w/v) agarose gels and stained with ethidium bromide. DNA fragments of Lamda phage digested with the restriction nucleases Hind III and Eco RI were used as standard for electrophoretic migration. **A:** unfertilized eggs; **B:** zygotes harvested 40 min p.i.; **C:** zygotes cultured in the presence of 3 ABA.

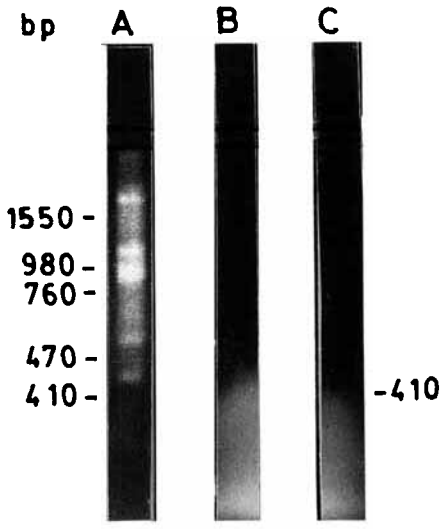

Fig. 2. DNA fragments derived from MNase digestion of nuclei isolated from unfertilized eggs and zygotes were electrophoresed in 1.9% agarose, 0.1% SDS gels. DNA fragments derived from phage ϕ X174 digested with restriction enzymes Hae III and Eco RI were run in parallel to each sample as DNA size markers. **A:** unfertilized eggs; **B:** zygotes harvested 40 min p.i.; **C:** zygotes cultured in the presence of 3 ABA.

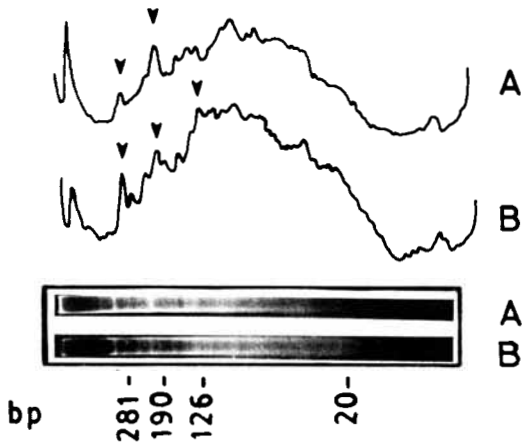

Fig. 3. Zygote nuclei were digested for 3 min with MNase. The DNA fragments derived from MNase digestion were purified and electrophoresed in 20% w/v polyacrylamide gels. DNA fragments from pBR322 digested with restriction enzymes Hae III were used as DNA size markers. **A:** zygotes harvested 40 min p.i.; **B:** zygotes cultured in the presence of 3 ABA.

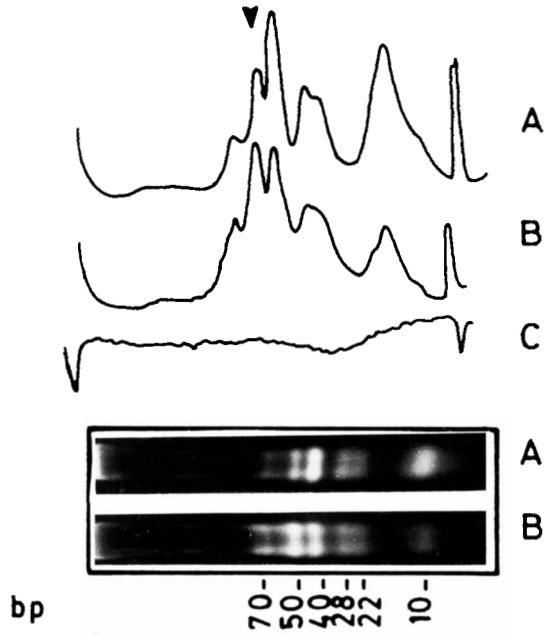

Fig. 4. Nuclei isolated from zygotes were digested for 10 min with MNase and the DNA fragments obtained as products of digestion were run in a 20% w/v polyacrylamide gel. DNA fragments from the pBR322 digested with restriction enzymes Hae III were used as DNA size markers. **A:** zygotes harvested 40 min p.i.; **B:** zygotes treated with 3 ABA from 3 min p.i.; **C:** protein free DNA.

To define more precisely the MNase digestion patterns of S phase chromatin, zygote nuclei were exposed to more extensive digestions and the products were subsequently analyzed until a clear pattern was obtained. As shown in Figure 4, a well-defined pattern of DNA fragments distributed under the size of a mononucleosome was obtained after 10 min of digestion. To confirm that this pattern of migration represents the interaction of DNA with proteins, and does not reflect recognition of specific DNA sequences, we compared these patterns with those obtained when protein free sea urchin sperm DNA was digested with MNase (Fig. 4C). As shown, MNase digestion of protein free DNA results in a smear of stained DNA fragments, indicating the absence of preferential cutting sites. These findings confirm that the MNase digestion pattern obtained from zygote nuclei is determined by protein-DNA interactions, although both control and 3 ABA treated zygotes show very similar patterns of digestion. A unique quantitative difference was found which affects the peaks located at 79 and 63 bp. This difference suggests that the normal interaction of DNA with nucleosomal proteins may be slightly modified by the inhibition of the poly(ADP-ribosylation) of CS histone variants by treatment of zygotes with 3 ABA.

DISCUSSION

In this report we demonstrate that during DNA replication a major fraction of chromatin is present as unfolded nucleosomes in nuclei of sea urchin zygotes. In contrast to the open structure of the zygote chromatin observed during the first S phase, the nucleosomes derived from the chromatin of unfertilized eggs are organized into polynucleosomes. The basis for this conclusion is provided by studies carried out with the most widely used experimental approach for detecting a nucleosomal organization in chromatin, the micrococcal nuclease (MNase) digestion patterns. MNase digestion studies are commonly used to investigate the boundaries of DNA incorporated into chromatin nucleoprotein particles [Gross and Garrad, 1988]. In this report we present direct evidence indicating that zygotes harvested during the first S phase exhibit a MNase digestion pattern of chromatin in which mononucleosomal particles are not evident as the major product, but discrete DNA fragments corresponding to a sub-nucleosomal array are found. Accordingly we postulate the presence of a major proportion of unfolded nucleosomes at the onset of S phase in sea urchin zygotes. This unfolded structure seems quite stable, because a very precise and defined pattern is obtained after extensive digestion of chromatin. However, it was also evident that

the S phase specific unfolded nucleosomes are further organized into discrete chromatin nucleoprotein particles. These particles should be structured by a protein network since they migrate as a discrete entity in agarose gels, despite the small DNA fragments, below the size of a single nucleosomal unit. Interestingly the whole nucleoprotein particle appears to be similar with respect to electrophoretic migration in both zygotes and unfertilized eggs. But, in contrast to zygotes, the sizes of DNA fragments found in egg derived particles are distributed as a ladder typical of polynucleosomes. The discrete nucleoprotein particles found in eggs and zygotes may represent specific features of chromatin domains. At present, the exact meaning of different domains of chromatin structure are not well understood, except for the chromatin loops bound to nuclear matrix that are postulated to be representative of a transcription unit [Huang and Spector, 1991; Carter et al., 1993; Xing et al., 1993].

From the results discussed above, it can be deduced that after fertilization there is a remodelling of nucleosomal structure that is temporarily correlated to the onset of the first DNA replication wave. This change will lead from a folded conformation inaccessible to the replication machinery in unfertilized eggs, to an open nucleosomal organization, with no steric hindrance to progress of the DNA replisome. The molecular mechanisms responsible for this change are still unknown.

On the basis of results presented in this report it can be inferred that the open-nucleosomal array found in sea urchin zygotes is not a consequence of DNA replication, since it is not dependent on the onset of S phase. The unfolded nucleosome structure is not modified if DNA replication is inhibited by decreasing the degree of poly(ADP-ribosylation) of nucleosomal proteins by treatment with 3 ABA. This finding is consistent with a previous demonstration that the poly(ADP-ribosylation) of CS histone variants is a pre-requisite and not a co-requisite for successful progress of the first DNA replication wave in sea urchin zygotes [Imschenetzky et al., 1991b, 1993a]. The principal difference observed in chromatin structure of control and 3 ABA treated zygotes was a quantitative modification in nuclease accessibility to intranucleosomal domains. The relevance of the observed intranucleosomal arrangement for DNA replication is difficult to interpret. It may be speculated that a specific open subnucleosomal structure may be needed for an ordered and sequential progress of the replication machinery through the template. This has also been suggested by Kornberg [1977]. Alternatively it may be speculated that a specific subnucleosomal structure may be crucial for the interaction of chromatin with macromolecules which mediate general organization of the nucleus during S phase. The nucleoskeleton, nuclear matrix, and nuclear scaffold have been implicated as principal components of nuclear organization by multiple experimental approaches [Cook, 1991; Getzenberg et al., 1991; Getzenberg, 1994]. There is increasing evidence for functional relevance of the nuclear matrix to the integration of physiological regulatory signals for DNA replication, recombination, as well as cell growth, transcription, and cell differentiation [Stein et al., 1994; Majumder and DePamphilis, 1994]. One can postulate the binding of several loops of unfolded nucleosomal thread to the nuclear matrix during S phase in sea urchin zygotes. Such a model is consistent with the transient association of different enzymes participating in DNA replication with the nuclear matrix during S phase [Jackson and Cook, 1986; Jackson et al., 1988].

It is well documented that cleavage cell cycles in animal species ranging from echinoderms through mammalians are characterized by rapid waves of DNA replication [Majumder and DePamphilis, 1994]. This high rate of DNA replication is mainly due to the recruitment of an increased number of DNA replication origins that are coordinately activated. The experimental data indicates that early embryos recognize at least five times more initiation sites than do differentiated cells of the same species [DePamphilis, 1993]. This finding has being interpreted as indicative of the absence of a requirement for specific origins of DNA replication in early cleavage cells. Alternatively, different sequences may function as putative origins of replication in these cells [Majumder and DePamphilis, 1994]. The results presented in this report of a remodelling of chromatin structure in S phase zygotes, support a significant contribution of chromatin organization to the high rate of DNA replication in cleavage cells. The observed open structure may be pre-requisite for S phase in cleavage cells and may represent a coordinate unfolding of nucleosomes in different chromatin domains. We also postulate that in vivo, this unfolding of nucleosomes is not strictly

related to a more extensive poly(ADP-ribosylation) of chromosomal proteins. Such a mechanism can not be totally dismissed since this post-translational modification is only partially inhibited in vivo at the 3 ABA concentrations used in our experiments, although it almost completely abolishes DNA synthesis [Imschenetzky et al., 1993a]. Another consideration is that the small nucleosomes present in cleavage stage sea urchins which are formed by CS histone variants [Newrock et al., 1978; Savic et al., 1981; Shaw et al., 1981; Chambers et al., 1983; Richards and Shaw, 1981] may be functionally related to the high rate of DNA replication. Although the precise molecular characterization of individual components of the CS histone variants remains to be determined, it has been previously demonstrated in the sea urchin *Tetrapygus niger* that the CS variants are immunologically unrelated to the somatic type histones found in pluteus larvas [Imschenetzky et al., 1993b]. These CS histone variants are specifically found as major components of nucleosomes only in unfertilized eggs and early cleavage cells; their representation decreased in whole chromatin as development advances. The decreased level of CS histone variants is accompanied by a decreased DNA replication rate in larval stages of development [Poccia, 1986]. These changes in histone composition are paralleled by a different population of nucleosomes that become increasingly heterogeneous during subsequent larval stages of development [Shaw et al., 1981; Savic et al., 1981]. The smallest of these nucleosomes is found in unfertilized eggs, where approximately 126 bp of DNA form the primary unit of chromatin structure [Imschenetzky et al., 1989]. Taken together, our results suggest that at least in sea urchins, the high rate of DNA replication which occurs during cleavage stages of development may also be determined by unique nucleosomes that contribute to a diffuse chromatin organization.

ACKNOWLEDGMENTS

We are very grateful to Drs. Gary Stein and Janet Stein from the University of Massachusetts USA for their suggestions, critical reading, and for correcting the English of this manuscript. Thanks also to Dr. Claus Von Holt for his helpful suggestion. Supported by grants 1930361 and 92-293 FONDECYT and from Universidad de Concepción, Chile.

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