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Relationship between basic proteins associated to DNA and replication during early development of Echinoderms

Relación entre proteínas básicas asociadas a DNA y replicación durante el desarrollo temprano de Echinodermos

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A close relationship between histone synthesis and DNA replication has been suggested for many biological systems. The presence of histonic proteins has been demostrated in practically all kinds of eukaryotic cells. During the clevage period of sea urchins it has been postulated that histones are not present in nuclei, and that only at blastula state they do associate with DNA.

Our results suggest that the failure to isolate histonic proteins from nuclei derived from cleavage cells might be caused by the presence in those cells of proteases activated by $NaHSO_3$. This compound has being widely used to inhibit proteolitic action in other biological systems.

By changing the method for chromatin isolation, we have been able to isolate basic proteins from nuclei of gametes, zygotes and 2-4 blastomeres.

These proteins behave like calf thymus histones in urea-acetic acid polyacrilamide gels, and they do not show great differences from proteins isolated from nuclei of blastula, gastrula, prism, and pluteus.

The electrophoretic patterns of basic proteins obtained from eggs and zygotes are practically identical, except for one protein moving like lysine-rich calf thymus histones. This protein appears in zygote nuclei at the beginning of the first replication wave.

In most eukaryotic systems a functional relationship between histone genes expression and DNA replication is suggested by the following facts:

a) Histone genes are transcribed and translated only during S phase of the cell cycle (1) (2).

b) If DNA synthesis is inhibited, a very rapid shut down of histone mRNA translation is observed on cytoplasmic polysomes, if protein synthesis is inhibited in G_1 period of the cell cycle, DNA replication slows down, to be finally arrested in the next S period (3) (4).

c) Chemical modifications of lysine histonic fraction have also been correlated with replication process: a phosphorylation of this fraction has being observed at the beginning of the replication wave (5) and DNA dependant ADP ribosylation has been reported to inhibit DNA synthesis.

Taking this data together, it seems clear that histones are closely related to DNA replication in eukaryotes (6).

During early cleavage stages of development most of the cellular activity is derived to DNA replication. As it is shown in Fig. 1 in this system DNA synthesis is not dependent on histone genes transcription as mRNA containing information for histones are already present in unfertilized eggs (7) and evidence has been presented that these types of messenger RNA are translated on small polysomes in cytoplasm.

However a relationship between DNA replication and histones synthesis is also present in this system: If DNA synthesis in inhibited a disagregation of small polysomes will occur, (8) this (polysomes, 200 S) has been shown to be the site of histones mRNA translation.

On the contrary, if protein synthesis is blocked, a slow down of DNA replication will be observed for the first S period and will be strongly inhibited for the second replication wave (9) (10).

The presence of histonic proteins in nuclei during cleavage stages of sea urchin had remain a controversial point. Several reports had shown that the main five types of histones are not associated with chromatin during cleavage stages, and that they would associate with DNA only at blastula stage (11) (12(.12).

This data would imply a very rapidly replicating chromatin associated with proteins different from histones.

We were, therefore, interested in studying acid soluble proteins present in gametes, zigotes, cleavage stages, and during early development of the sea urchin *Tetrapygus niger*.

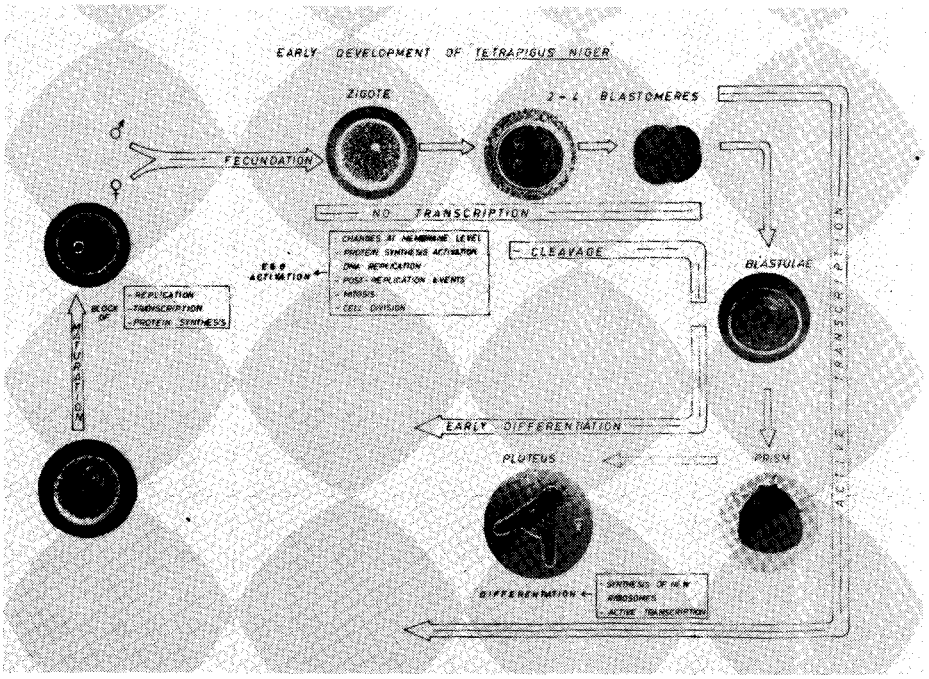

Fig. 1. Molecular events in sea urchin early development.

MATERIAL AND METHODS

1. *Handling of sea urchins*

Teatrapigus Niger were collected before each experiment, spawned by gentle shaking gonads in sea water. Sperm were obtained dry and diluted immediately before each insemination. Zygotes and embryos were obtained as described elsewhere. Only batches containing more than 95% syncronization were used.

2. *Chromatine purification and histone extraction*

(All steps were performed between 0°C and 4°C). Eggs or embryos were collected by pelleting at 30 × g for 2 min, suspended in 1.5 M dextrosa and centrifuged at 800 × g for 15 min. several times. Eggs/embryos pellet was homogenized in 10 volumes of EDTA 50 mM pH = 6.0 with Potter Evelhjenm homogenizer, filtered through a placton network (40 μ) and crude nuclear pellet was obtained at 1950 × g for 15 min. Nuclear pellet was suspended twice in 1M sucrose, 50 mM EDTA pH 6.0, Tritón x −100 0.2% and pelleted at 2000. g.

The chromatin pellet was then washed 3 times with EDTA pH 6.0 50 mM; 5 mM and 1 mM respectively. Histones were extracted from chromatin by overnight stirring in 0.4 N H_2SO_4. Acid insoluble material was centrifuged out, and the acid soluble material was concentrated and dialyzed against Acetate buffer 0.04 M pH = 2.9 and analyzed by acetic acid-Urea polyacrylamide electrophoresis. Or dialyzed against water and freeze dried.

3. *Electrophoresis in polyacrilamide gels*

Was performed according to Vorobyev. (13).

4. *Tryptophan determination*

The luminisence of proteins solutions was compared with that of tryptophan at 345 nn. The excitation of luminisence was performed by a monochromatic light at 280 nm (14).

RESULTS

ANALISIS OF ACID SOLUBLE PROTEINS ISOLATED FROM CHROMATINE OF UNFERTILIZED EGGS AND EARLY CLEAVAGE STAGES BY POLYACRILAMIDE GEL ELECTROPHORESIS

Most of the media commonly used to isolate chromatin from different tissues including sea urchin early embryos (11) (12) contain $NaHSO_3$ 50 mM to prevent proteolytic degradation of histones. However, when chromatin of unfertilized eggs and early cleavage stages is isolated in the presence of $NaHSO_3$ (50 mM), an important proteolysis of acid soluble proteins associated with chromatin is obtained (15). These basic proteins are easily extracted from chromatin under conditions that will completely prevent proteolitic degradation (described in methods).

In Fig. 2 a polyacrilamide gel electrophoretic analysis of acid soluble proteins extrated from chromatin isolated in a medium containing sodium bisulfite (11), are compared, with those obtained under conditions described in methods.

If proteolytic action is not prevent, one major fast moving band, corresponding to peptides produced by hidrolysis is found. However if chromatin isolation and acid protein extracts are peformed as described in methods, several electrophoretic bands are obtained corresponding to acid soluble proteins that are associated with chromatin in unfertilized eggs and early zygotes.

The electrophoretic patterns of chromatin acid soluble proteins from unfertilized eggs, zygotes and 2 blastomers are compared in Fig.

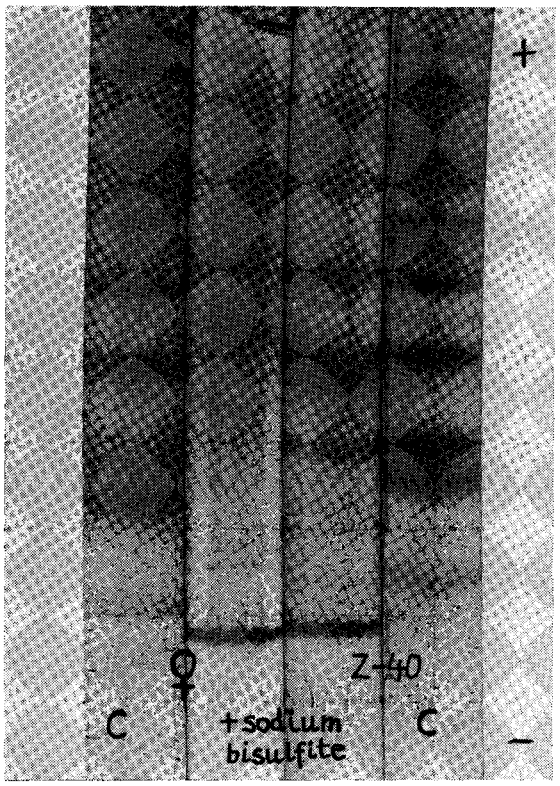

Fig. 2. Electrophoretic separation of acid soluble proteins, extracted from chromatin isolated by the method of Johnson and Hnilica and as described in methods, (11) (♀) Basic proteins extracted from chromatin of unfertilized eggs. (Z-40)' Basic proteins extracted from chromatin of zygotes, 40 minutes post insemination.

3 with those obtained with histones extracted from sea urchins embryos in blastula, gastrula and pluteus stages of development, calf thymus comercial histones and with sperms histones.

As shown in Fig. 3, in unfertilized eggs and during early cleavage stages, there are acid soluble proteins associated with chromatin that comigrate with the five major histone fractions present in later stages of development and in calf thymus.

Histones do not contain tryptophan, thus tryptophan content of the acid soluble proteins extracted from eggs and early embryos may be regarded as a criteria of their purity and of their similarity to typical histones described for other eukaryotic cells.

As a means of further identifying the acid soluble protein extrated from chromatin of eggs and early cleavage stages, a tryptophan determination was performed as indicated in methods.

The acid soluble proteins extracted from unfertilized eggs, zygotes and 2 blastomers stage do not contain a detectable amount of tryptophan. Neither do the proteins extracted from sperm, blastula, gastrula, prism and pluteus.

RELATIONSHIP BETWEEN HISTONES ASSOCIATED WITH CHROMATIN AND DNA REPLICATION

In order to determine the onset of DNA synthesis in Tetrapygus niger embryonic system, incorporation of exogenously supplied H^3 thymidine into acid insoluble fraction (DNA) was performed at different times after fertilization.

As shown in Fig. 4 between 30 and 40 minutes post insemination, a clear rise of radioactivity is obtained in the acid insoluble fraction, H^3 thymidine incorporation increase up to 100 minutes post insemination.

During the interval between 30 and 100 minutes after insemination, chromatin of the zygote will be replicating. In order to determine histone content of chromatin during replication as apposed to histone present in chromatin from gametes (non replicating chromatin), histones were extracted from zygotes 40 minutes post insemination, as indicated in methods.

A comparison of histonic proteins present in zygotes 40 minutes after fertilization (Z40) with histones present in gametes is shown in Fig. 5.

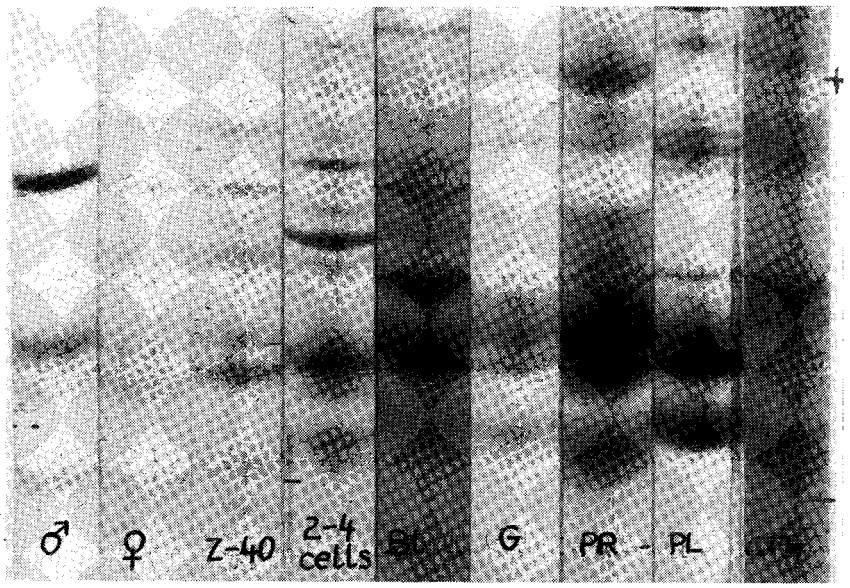

Fig. 3. Electrophoretic separation of acid soluble proteins extracted from chromatin isolated as described methods from:

(♀) unfertilized eggs;
(♂) sperm;
(Z-40) zygote, 40 minutes after fertilization;
(2-4 cells) 2-4 blastomere stage;
(Bl) blastula; (G) gastrula;
(Pr) Prism and (Pl) Pluteuns.

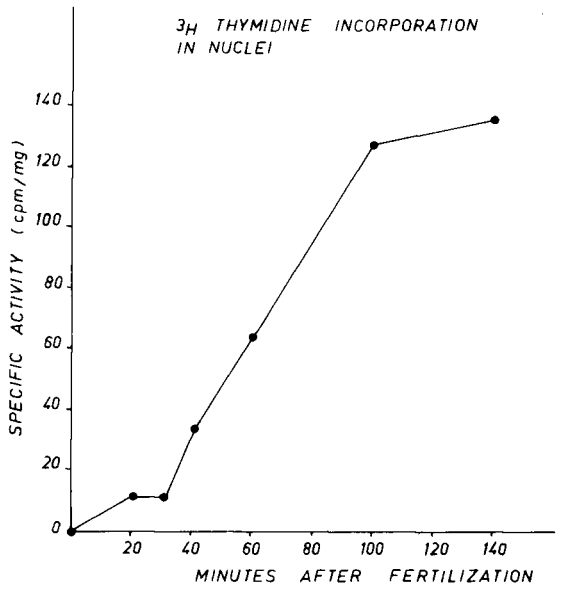

Fig. 4. Incroporation of H^3 thymidine into DNA in *Tetrapygus niger* Zygotes. The sperm suspension was added at zero minute.

As shown in Fig. 5 all the acid soluble proteins presented in unfertilized eggs are also present in zygote during replication period.

One of the histonic fractions from sperm having a electrophoretic mobility of a moderately lysine rich histone is not found in zygotes.

A unique zygote component, migrating in lysine rich histone region, is distinct from the basic proteins present in unfertilized eggs and sperm.

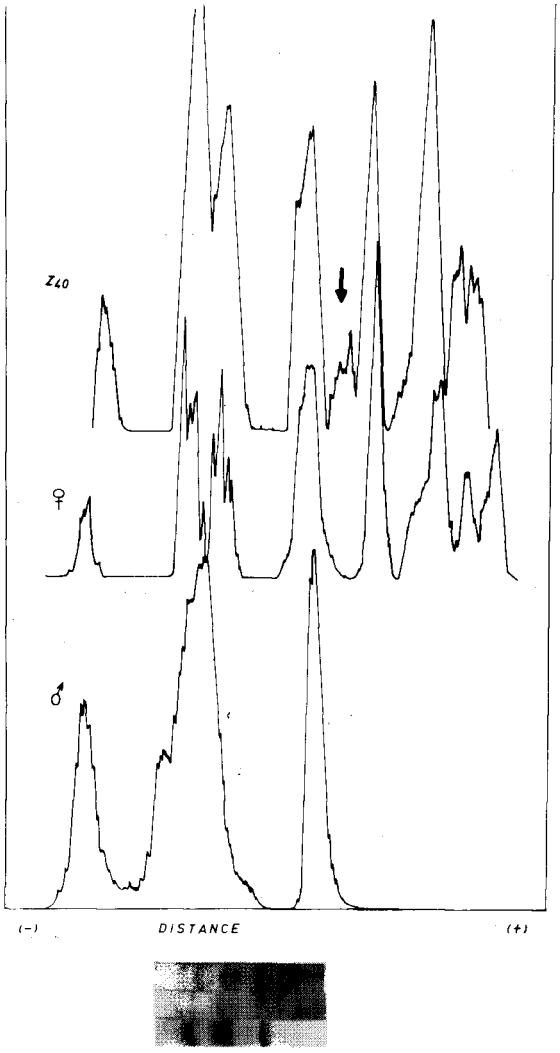

Fig. 5. Spectrophotometric scans of Urea-Acetic acid electrophotogram of acid soluble proteins, extracted from chromatin of sperm (♂); unfertilized eggs (♀) and zygote 40 minutes after fertilization (Z-40).
The arrow points to the band present only in zygotes.

DISCUSSION

The results presented in this report show that basic proteins present in chromatin of unfertilized eggs and early cleavage stages of development of *Tetrapygus niger* are histonic proteins. The evidence may be summarized as follows:

1. They are soluble in 0.4 N, H_2SO_4, therefore they meet the criterion of solubility of histones.

2. They do not contain tryptophan, thus they are not contamined by acidic nuclear proteins or citoplasmic proteins.

3. Their electrophoretic mobilities are very similar to histones extracted from latter stages of development (blastula, gastrula and pluteus) and to histones extracted fron calf thymus.

Histones have been reported to be absent from sea urchin chromatin before blastula stage (11) (12). Detection of acid soluble proteins having electrophoretic mobilities similar to some histonic fractions from nuclei of *Strongylo centrotus Purpuratus* (16) and *Arbacia Punctulata* has also been presented (17) However in both reports, acid soluble proteins were extracted from whole nuclei. In both studies, no determination of purity was performed, and no controls of proteolytic degradation are presented. The failure to isolate a pure histonic fraction from sea urchin

unfertilized eggs and during early cleavage, is probably due to the use of $NaHSO_3$ as a protector against proteolitic degradation. This compund activates proteolytic enzymes 400%, in the conditions used for chromatin isolation previous studies (11) (12).

The electrophoretic patterns of acid soluble proteins, extracted from zygotes at the beginning of the first replication wave is practically identical to acid soluble proteins present in unfertilized egg, except for one electrophoretic band migrating in the region of somatic lysine rich histones.

The relationship between this protein and the DNA replication process, should be further investigated.

One of the sperm histones migrating in the slightly lysine rich histone region is absent in zigote chromatin. This fact suggests a possible hidrolysis of this fraction during the time of male pronucleus formation.

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RESUMEN

Una estrecha relación entre síntesis de histonas y replicación de DNA ha sido sugerida para muchos sistemas biológicos. La existencia de proteínas histónicas ha sido demostrada en prácticamente todas las células eucarióticas.

Durante las etapas de clivaje de erizos de mar se ha postulado que las histonas no estarían presentes en el núcleo y que sólo al estado de blástula se asociarían a DNA.

Nuestros resultados sugieren que la incapacidad para aislar histonas a partir de núcleos de embriones en clivaje, puede deberse a la presencia de proteasas activadas por $NaHSO_3$ en estos estados de desarrollo. Este compuesto ha sido ampliamente utilizado en otros sistemas biológicos como protector de proteólisis.

Al cambiar el método de purificación de cromatina, hemos podido aislar proteínas básicas a partir de gametos, zigotos y 2-4 blastomeros.

Estas proteínas presentan un pattern electroforético semejante a histonas de timo de ternera, blástula, gástrula, prisma y pluteus.

Los patterns electroforéticos de las proteínas básicas obtenidas de ovocitos y zigotos son prácticamente idénticos, a excepción de unas proteínas que migra en la región de histonas ricas en lisina de timo de ternera.

Esta proteína aparece en el núcleo de zigotos al comienzo de la primera fase replicativa.