Replication timing: histone genes replicate during early S phase in cleavage-stage embryos of sea urchin

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Received 17 June 1986; Revised and Accepted 3 November 1986

ABSTRACT
Newly synthesized DNA was separated from the bulk of the DNA by pulse-labeling with BUdR and centrifugation in an alkaline CsCl buoyant density gradient. The content of histone gene in the newly synthesized DNA was determined by DNA dot hybridization. The gene contents in DNA replicated during the early half of S phase and during the whole S phase were compared. Results showed that histone genes were replicated during the first half of the S phase in embryos in the early cleavage stage.

INTRODUCTION
In embryos at early cleavage stages, the cells divide synchronously and very rapidly, possibly by a specific mechanism of cell proliferation differing from that of the more slowly dividing somatic cells. Experiments on embryonic cells of Drosophila melanogaster and Triturus have suggested that the very rapid DNA replication in embryonic cells is guaranteed not by increase in the rate of DNA chain growth, but rather by increase in the number of available initiation sites (1, 2). For determination of the mechanism underlying the temporal and successive activation of initiation sites of chromosomal DNA of cleaving embryos, the mode of replication of a DNA fragment of known characters must be studied. Accordingly, we have started to study how DNA fragments (approximately 7 kb) coding for histone proteins are replicated.

The reiterated histone genes of the sea urchin, isolated by the classic centrifugation technique (3), and by cloning (4, 5) have been the object of intensive investigations at a gross structural level by restriction mapping and molecular RNA/DNA hybridization. Because of the conservativeness of the amino acid sequences of histones, the histone genes of the Japanese
sea urchins *A. crassispina* and *H. pulcherrimus* can be detected by use of a cloned sea urchin (*P. miliaris*) histone gene (clone h22 (6)) as a probe. The copy numbers and sizes of the histone genes of *A. crassispina* and *H. pulcherrimus* are similar to those of other sea urchins. These results will be reported elsewhere with mapping data on the cloned histone gene of *A. crassispina* (manuscript in preparation).

The highly reiterated histone genes that code for "early class histones" are rare genes that are active during the period from meiotic maturation to the blastula stage (7, 8). In cultured mammalian somatic cells, active genes, such as "housekeeping" genes, replicate during the first half of the S phase, whereas sequences that are not expressed, such as satellite DNA and inactive X chromosomal DNA in mammalian females, replicate during the last half of the S phase (9, 10, 11). This bi-phasic nature of DNA replication in eukaryotic cells allows the distinction of two classes of replication units ("replicons"), early and late (12). In human cells in which histone genes are actively transcribed, it is reported that histone genes are replicated during the early half of S phase (13). In the genome of the sea urchin also, the histone gene clusters are expected to replicate early during the cleavage stage. However, the S phase is very short in sea urchin embryos at this stage and so the times of replication timing of genes are difficult to determine during early development. In the present study, we determined, for the first time, the time of replication of histone genes in cleavage stage embryos. We found that they replicate within the first half of the S phase in sea urchin embryos in the early cleavage stage.

**MATERIALS AND METHODS**

**Culture of Sea Urchin Embryo**

Eggs and sperm of the sea urchins *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* were obtained by the conventional KCl method. The unfertilized eggs were filtered through nylon mesh, washed twice with artificial sea water and suspended at a concentration of about 10,000 eggs/ml in artificial sea water. Then ³H-deoxyadenosine (1μCi/ml) was added, followed ten mi-
nutes later by 1/100 volume of 0.1M aminotriazole. The eggs were then fertilized with diluted sperm and cultured with gentle stirring at 20°C. The suspended embryos showed highly synchronous cleavage for several divisions.

Measurement of DNA Synthesis
DNA synthesis by fertilized eggs was assayed by measuring the incorporation of the radioactivity of \(^{3}H\)-deoxyadenosine into DNA. For this, 1 ml samples of the embryo culture were taken at appropriate intervals and mixed with 2 ml of 10% trichloroacetic acid. Removal of acid-soluble radioactivity from the eggs and determination of acid-insoluble radioactivity were performed as described previously (14).

Preparation of Radiolabeled Histone DNA Probes
The cloned repetitive histone gene unit (clone h22) of the sea urchin *P. miliaris* (6) was a generous gift from Dr. Max L. Birnstiel (Institut für Molekularbiologie II, der Universität Zürich). In this clone, a HindIII digested fragment consisting of one cluster of histone genes is inserted into the HindIII site of the vector plasmid pCR 1. Uniform labeling of DNA by nick translation was performed as described by Rigby et al. (15).

Preparation of Genomic DNA
Genomic DNA was obtained from the sperm of *A. crassispina* and *H. pulcherrimus* by the method of Kedes (4).

Preparation of lambda phage DNA
Lambda phage DNA was prepared from phage (\(\lambda\) CI 857) transfected *E. coli* (W3110) by the method of Maniatis et al. (16).

Isolation of Newly Synthesized DNA from Cleavage Stage Embryo
The newly synthesized DNA was labeled with 10 \(\mu\)M 5-bromo-2'-deoxyuridine (BUDR, Sigma) which was added to cultures of embryos in the S phase. After labeling, 1/100 volume of sodium azide solution (100mM) and an equal mass of crushed frozen sea water were added to the cultures to stop DNA synthesis quickly. The embryos were harvested by centrifugation (8,000 rpm, 1 min, at 4°C), and nuclei were isolated by a slight modification method of Chambers et al. (17) as follows. The harvested embryos were suspended in 25mM EDTA-IG buffer [0.1 M sodium phosphate buffer (pH 6.0) containing 0.1 M lithium chloride]
by vigorous pipetting, and centrifuged at 3,000 rpm for 5 min at 4°C. The pellet was washed with the same solution once more. The embryos were washed twice more with IG buffer and homogenized by 10 to 20 strokes in a Dounce homogenizer. The homogenate was centrifuged at 3,000 rpm for 10 min, and the pellet was washed with IG buffer. The washed pellet was suspended in 100 mM Tris-HCl (pH 8.5), 10 mM EDTA, 1% Sarkosyl and 2mg/ml of Actinase E (The Actinase E was preincubated at 37°C for 1 hr before use). The suspension was incubated at 37°C for 2 hours, and then 1/4 volume of 5 M sodium perchlorate was added and the suspension was extracted twice with phenol, once with phenol-chloroform (1:1), and once with chloroform. DNA was precipitated from the extract with 2 volumes of ethanol, dissolved in 0.1 N NaOH - 0.05% Sarkosyl and denatured by heating in a boiling water bath for 10 min. The DNA was then mixed with 5.2 volumes of saturated cesium chloride (CsCl) in the same solution, and the refractive index of the solution was adjusted to 1.4060 at 20°C with NaOH-Sarkosyl solution or with saturated CsCl solution. The solution was centrifuged at 33 k rpm for 60 hours at 20°C in Beckman type 50 Ti rotor, fractions were collected from the bottom of the tube, and their radioactivities were determined. Newly synthesized DNA and bulk DNA were pooled separately, dialyzed against TE buffer (10 mM Tris, 1 mM EDTA) and precipitated with ethanol.

Determination of Genomic Contents of Histone Genes

The relative concentrations of histone genes in the sea urchin genome were determined by dot hybridization (18). The DNA precipitated with ethanol was dissolved in 0.25 N NaOH, denatured by heating in a boiling water bath for 10 min, and mixed with an equal volume of 2 M ammonium acetate. Volumes of 5 ul of samples, standard DNA (h22) solution and negative control DNA (lambda phage DNA) solution were spotted on nitrocellulose membrane filters (BA 85, Schleicher & Schuell GmbH, W. Germany) fixed in a filtering apparatus (Minifold, Schleicher & Schuell GmbH, W. Germany). The filters were then washed once with 1 M ammonium acetate and once with 4 X SSC, and baked in vacuo at 80°C for 2 hours. DNA on the filters was hybridized with the radioactive probes in 6 X SSC solution containing 20 mM
sodium acetate buffer (pH 6.5), 1 X Denhardt solution, 50 mM formamide (Merck), 100 µg/ml of heat denatured salmon sperm DNA and 10% dextran sulfate (Pharmacia) at 42°C for 14 hours (19). The filters were then washed with 0.1 X SSC-0.1% SDS at 42°C and autoradiographed with Kodak X-Omat AR film for 3 to 7 days. Spots located from the autoradiograms were cut out and their radioactivities of ³²P and ³H were determined in a liquid scintillation counter.

RESULTS

Newly synthesized DNA was labeled with BUdR during the early half of the S phase or throughout the S phase as shown in Fig. 1. The labeled DNA was separated from the bulk DNA by equilibrium density gradient centrifugation in CsCl as described in MATERIALS AND METHODS. As shown in Fig. 2, the BUdR-labeled DNA was separated from the bulk DNA in this way. For deter-

![Graph](data:image/png;base64,imagedata)

**Fig. 1.** Time course of DNA synthesis in fertilized eggs of *A. crassispina*. DNA synthesis of fertilized eggs was assayed by the incorporation of ³H-deoxyadenosine into DNA as described in MATERIALS AND METHODS. For density-labeling of newly replicating DNA during the third S phase, bromodeoxyuridine (10 µM) was added to the embryo culture after completion of the second S phase (105 min after fertilization, as indicated in the figure). Half the embryos were harvested at the half point of the third S phase (A) and the remainder were harvested at the end of the third S phase (B), as indicated by arrows in the figure.
Fig. 2. Separation of BUdR-labeled, newly replicated DNA from bulk DNA by CsCl buoyant density centrifugation. DNA replicated during the early half (A) or during a full round (B) of the third S phase was extracted from the embryos and centrifuged in an alkaline CsCl gradient as described in MATERIALS AND METHODS. Then fractions were collected from the bottom of the tube and aliquots of each fraction were used to determine the amount of \(^{3}H\)-deoxyadenosine in DNA (o-o). The refractive index of each fraction was measured with a refractometer (e-e). Centrifugation was from right to left. The peak fractions of radioactivity of BUdR-labeled DNA (Heavy) and bulk DNA (Light) were each collected as indicated.

mination of the histone gene contents of these two fractions of DNA (newly-synthesized DNA and bulk DNA), equal amounts of BUdR-labeled \(^{3}H\)-DNA and bulk \(^{3}H\)-DNA were serially diluted, dotted on nitrocellulose filters, hybridized to \(^{32}P\)-h22 probe and autoradiographed (Fig. 3). For confirmation of the accuracy of measurements, genomic DNA from sperm of the sea urchin *A. crassispina* or *H. pulcherrimus*, h22 DNA and lambda phage DNA were also serially diluted and dotted on the same sheet of filter. Results showed that \(^{32}P\)-h22 did not hybridize to lambda DNA and that significant amounts of \(^{32}P\)-h22 hybridized to sperm DNA and h22 DNA. From the amounts of DNA hybridized, we estimated that the histone gene occupies about 0.04% of the total genomic DNA of the sea urchin *A. crassispina*. A similar value was obtained for *H. pulcherrimus* (data not
Fig. 3. Histone gene content of newly replicated DNA. The DNA newly replicated during the early half of the third S phase (Half, H) and during a full round of the third S phase (Full, H) were isolated by buoyant density centrifugation as shown in Fig. 2, and each DNA fraction was serially diluted and dotted on a nitrocellulose filter. The bulk DNA fractions (Half, L; Full, L) of samples were also diluted and dotted on the filter. The dilutions are shown in the figure. The tritium counts of spots at the same dilution were similar. Sperm DNA of A. crassispina (sperm DNA) and h22 DNA (h22) were used as positive controls and DNA of lambda phage (λ-DNA) was used as a negative control. These control DNAs were also serially diluted and dotted on the filter. Then the filter was hybridized with ³²P-labeled h22 clone and autoradiographed as described in MATERIALS AND METHODS. The amounts of these standard DNAs are indicated in the figure. The amounts of dotted ³H-DNA and hybridized ³³P-probe were determined by liquid scintillation counting. The radioactivities of the hybridized probe per unit amount of dotted DNA were calculated and are shown in Table 1, exp. no. 1.

This value is somewhat less than those obtained for P. miliaris and other sea urchins (20) (see DISCUSSION). The amount of probe hybridized was quantified by determining the radioactivities in spots cut out from the filter. Since the genomic DNA had been labeled with ³H-deoxyadenosine in vivo, the total amount of DNA on the nitrocellulose filter could be determined directly by the tritium counting. Thus the histone gene content of DNA dotted on the filter could be expressed by the value for ³³Pcpm/³Hcpm, as shown in Table 1. Because the light DNA fraction consisted of bulk DNA that was present before initiation of the third S phase, the light fractions from the half-replicated and the fully-replicated DNA were considered to re-
Table 1. Histone gene contents of newly replicated DNA.

<table>
<thead>
<tr>
<th>Exp.No. (a)</th>
<th>DNA fraction (c)</th>
<th>Histone gene/DNA ((^{32})P-cpm/(^{3}H)-cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Half replicated (b)</td>
<td>Fully replicated (b)</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>Heavy</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>0.640</td>
<td>0.720</td>
</tr>
<tr>
<td></td>
<td>0.483</td>
<td>0.750</td>
</tr>
<tr>
<td>2</td>
<td>0.112</td>
<td>0.227</td>
</tr>
<tr>
<td></td>
<td>0.060</td>
<td>0.213</td>
</tr>
<tr>
<td>3</td>
<td>0.071</td>
<td>0.081</td>
</tr>
<tr>
<td></td>
<td>0.044</td>
<td>0.073</td>
</tr>
</tbody>
</table>

(a) Experiment 1, 2 and 3 are performed using the embryos of *A. crassispina* at S3, *H. pulcherrimus* at S2 and *H. pulcherrimus* at S3, respectively (see RESULTS).
(b) Half replicated DNA represents DNA isolated at the half point of the S phase. Fully replicated DNA represents DNA isolated at the end of the S phase.
(c) The heavy fraction represents newly-replicated DNA density-labeled with BUDR, and the light fraction represents the bulk of the DNA.

Present the template strand. Therefore, the contents of histone gene in these two fractions should be equal. As shown in Table 1, the histone gene contents of the light DNA fractions in the half replicated DNA and in the fully-replicated DNA were found to be almost equal. On the other hand, the histone gene content in the DNA fraction replicated in the early half of the third S phase of *A. crassispina* (Table 1, Exp. No. 1, column for Half replicated, Heavy) was significantly higher than that in the DNA fraction replicated throughout the third S phase (column for Fully replicated, Heavy). This suggests that almost all the histone genes replicate during the early half of the S phase. Similar results were obtained in S2 and S3, as shown by the results for embryos of an other species of sea urchin, *H. pulcherrimus* (Table 1, Exp. Nos. 2 and 3).

At the end of the S phase, the content of histone genes in the newly replicated DNA (heavy fraction) should be equal to that
in the bulk DNA (light fraction). However, since the histone gene contents are expressed as $^{32}\text{P} / ^3\text{H}$ ratios in Table 1, very high $^{32}\text{P} / ^3\text{H}$ ratios were obtained in the light DNA fractions of both half and fully replicated DNA, due to the presence of the unlabeled genomic DNA derived from the eggs and sperm.

DISCUSSION

Histone genes of the sea urchins, A. crassispina and H. pulcherrimus

The numbers of copies of the histone gene per genome, determined with the h22 probe of P. miliaris, were essentially similar in A. crassispina and H. pulcherrimus (approximately 70 copies per haploid genome). This value is somewhat less than the values reported for other species of sea urchin (20). We estimated, using a cloned histone gene of the same species, that the haploid copy number of the histone genes in A. crassispina is approximately 200. Therefore, this value could be an underestimate due to incomplete sequence homology between the spacer regions of histone gene of P. miliaris and Japanese sea urchins.

Replication timing of histone genes during the early cleavage stage

The histone gene contents of the DNA segments replicating in the early half of the S phase were compared with those of the DNA replicated throughout the S phase. The strategy of our experiment was based on the following assumption. If histone genes replicate randomly throughout the S phase, the contents of the genes per unit DNA in half replicated DNA and fully replicated DNA should be equal. If the genes replicate during the late half of the S phase, the heavier BUdR-substituted DNA fraction of the half replicated DNA, replicated only during the early half of the S phase, should not hybridize with the probe. On the contrary, if the genes replicate during the early half of the S phase, the gene content of the heavier fraction should be twice as much in the half replicated DNA as in the fully replicated DNA. The present results clearly showed that the histone gene content of the heavier fraction was significantly more in half replicated DNA than in fully replicated DNA. This was clearer
in experiments 2 in which the histone gene content in the heavier fraction was about twice as high in the half replicated DNA as in the fully replicated DNA. It is concluded therefore, that the histone gene replicates during the early half of the S phase in sea urchin embryos in the early cleavage stage.

One explanation proposed for the very quick DNA replication during the cleavage stage is that embryonic DNA may contain far more replication origins than somatic DNA (1, 2). The average size of the replicon in cleavage stage embryos has been determined by electron-microscopy to be about 0.7 kb (21). This size corresponds to about 1/10 of that of the total histone gene clusters (7 kb) which are repeated tandemly in the sea urchin genome (3, 4, 5). Therefore, there may be more than one replication origin in each of these clusters. Our results suggest that the replication origin in these histone gene clusters may be activated very early in the S phase and that replication of these regions is completed during the early half of the S phase.

Because of the large pool of deoxyribonucleoside triphosphates in the embryos in the early cleavage stage, as reported by Gourlie and Infante (22) and Mathews (23), density-labeling of DNA could not be achieved in a shorter period than that used in the present study.

ACKNOWLEDGMENTS

The authors thank Dr. Max L. Birnstiel (Institut für Molekularbiologie II der Universität Zürich) for a generous supply of the cloned sea urchin histone gene h22. Thanks are also due to the members of the Laboratory of Microbial Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo for their valuable suggestions on the experimental procedure, and to the staff of the Research Institute of Fisheries of Fukushima Prefecture, Iwaki, Fukushima, for a generous supply of sea urchins.

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