Nuclear distribution of proliferating cell nuclear antigen (PCNA) in fertilized eggs of the starfish Asterina pectinifera

Akira Nomura
Department of Zoology, Faculty of Science, Kyoto University, Kyoto 606, Japan
Present address: Tateyama Marine Laboratory, Ochanomizu University, Koh-yatsu, Umi-no-Hoshi, Tateyama, Chiba, 294-03, Japan

SUMMARY

Previous studies (Nomura et al. (1991) Dev. Biol. 143, 289-296 (1993) Dev. Biol. 159, 288-297) determined the time of DNA replication period (S phase) in starfish eggs fertilized either during or after oocyte maturation. Here proliferating cell nuclear antigen (PCNA) localized within nuclei of starfish eggs was detected with an anti-PCNA human antiserum. Using a confocal laser scanning microscope, a three-dimensional structure of the PCNA region was analyzed.

In eggs fertilized during maturation, PCNA started to localize within the nuclei at the same time as the initiation of the first S phase. During the S phase, the distribution of localized PCNA in a three-dimensional view coincided with the chromatin distribution. After the S phase, PCNA remained localized within the nuclei, but its distribution no longer coincided with the chromatin distribution.

In eggs fertilized after maturation, however, PCNA started to localize within the female pronuclei about 10 minutes ahead of the first S phase. Localized PCNA occupied only a limited region of the nuclei without diffusing over the whole nuclear area. Chromatin distributed around the peripheral region of the nuclei mostly outside the PCNA region. When the first S phase was initiated, the chromatin distribution became coincident with the PCNA region. Later behavior of PCNA was the same as that of the eggs fertilized during maturation. The precocious localization of PCNA in those eggs fertilized after maturation simply demonstrates that the ‘postactivation process’ for preparing DNA replication is triggered by fertilization and PCNA localization and S phase are sequentially initiated with a time-lapse. On the other hand, the simultaneous occurrence of them seen in those eggs fertilized during maturation indicates that the postactivation process must be going on in parallel with the maturation process.

Key words: starfish embryo, cell cycle, S phase, BrdU, PCNA, CLSM, three-dimensional reconstruction

INTRODUCTION

Starfish oocytes resume their maturation by 1-methyladenine (1-MA) treatment, and complete it without any arrest, followed by formation of the female pronucleus (Fig. 1A). The absence of ‘metaphase arrest’ is a characteristic feature of starfish oocytes. After the germinal vesicle breakdown (GVBD), they can be fertilized at any stage of their maturation (Fig. 1B,C). Thus, both oocytes and eggs are fertilizable. Irrespective of the time of fertilization, later development is apparently normal. If the eggs are fertilized after the completion of maturation and the formation of female pronuclei, DNA replication is initiated 30-50 minutes after fertilization (Nomura et al., 1991). This indicates that the DNA replication system of starfish will be established during this period of 30-50 minutes beginning from fertilization. The process for preparing DNA replication was called the ‘postactivation process’ by Nomura et al. (1991) (Fig. 1C). In contrast, if eggs are fertilized before polar body formation, DNA replication immediately follows pronucleus formation (Nomura et al., 1991). This means that the postactivation process must be going on and completed in parallel with the maturation process (Fig. 1B). From now on, eggs fertilized before polar body formation will be called ‘early fertilized eggs,’ and the eggs fertilized after the completion of the maturation process will be called ‘late fertilized eggs.’

Proliferating cell nuclear antigen (PCNA) has been a well-known protein essential for DNA replication in many types of cells (Tan et al., 1986; Bravo et al., 1987; Prelich et al., 1987; see also Fairman, 1990, for review). Association of PCNA with nuclei during the DNA replication period (S phase) was first found in mouse NIH 3T3 cells fixed with methanol: PCNA was detected in the nuclei only during S phase, and it was easily extracted from the nuclei during phases other than S phase (Bravo, 1986; Nakamura et al., 1986; Fairman et al., 1988). PCNA was finally identified as an auxiliary protein of DNA polymerase δ (Bravo et al., 1987; Prelich et al., 1987). It is now certain that PCNA binds to the replication fork, and is an indispensable protein molecule for the DNA replication system (see van der Vliet, 1989; Laskey et al., 1989, for review).

In 1989, Takasaki and his colleagues found that a human antiserum from an auto immune patient recognized not only human PCNA but also higher plant PCNA (Suzuka et al.,...
MATERIALS AND METHODS

Cell culture and BrdU labeling

The starfish Asterina pectinifera were collected on the coast of Wakasa Bay and Bohso Peninsula, Japan, and stored in aquaria in Tateyama Marine Laboratory, Tateyama.

As described in a previous paper (Nomura et al., 1991), the oocytes were: (i) induced to mature with 1 µM 1-methyladenine (1-MA); (ii) inseminated; and (iii) timed visually for their developmental events. Germinal vesicle breakdown (GVBD), and first and second polar body (PB1 and PB2) formation occurred, respectively, about 20 minutes, 60-70 minutes and 90-100 minutes after 1-MA treatment at 20°C (cf. Figs 2, 3). They were inseminated at 40 minutes or 2 hours after the 1-MA treatment. Eggs inseminated 40 minutes after the 1-MA treatment are the 'early fertilized eggs' and eggs inseminated 20° C (cf. Figs 2, 3). They were inseminated at 40 minutes or 2 hours after the 1-MA treatment. Eggs inseminated 40 minutes after the 1-MA treatment are the 'early fertilized eggs' and eggs inseminated 2 hours after 1-MA are the 'late fertilized eggs.' Oocytes, eggs, and embryos were cultured in a thermostatic bath at 20°C.

The fertilization membrane of the eggs was removed 5-10 minutes after the insemination as described in a previous paper (Nomura et al., 1993). Samples of eggs taken from the culture dish every 4 minutes were labeled with bromodeoxyuridine (BrdU; Sigma Chemical Co., St Louis, MO) for 3 minutes in seawater containing 10 mM BrdU and 0.05% Triton X-100 (Sigma). After the 3 minute labeling, they were fixed with methanol for 30 minutes at room temperature. They were then rinsed with phosphate-buffered saline (PBS) at pH 7.3, three times. Some eggs in each sample were processed for detection of the BrdU, and the other eggs were processed for staining for PCNA.

Immunocytological procedures

BrdU incorporated into the nuclei of eggs was detected with an anti-BrdU monoclonal mouse antibody to time the length of S phase. About 100 eggs were attached to a glass slide coated with poly-L-lysine (Sigma). The eggs were incubated in a drop of a reagent or an antibody in a moist chamber according to the following order: (i) 2 M HCl for 1 hour to denature the DNA; (ii) monoclonal mouse antibody against BrdU (Becton Dickinson Immunocytometry Systems, San Jose, CA) diluted to 1:6 with PBS for 2 hours; (iii) biotinylated sheep monoclonal antibody against mouse Ig (Amersham, Buckinghamshire, England) diluted to 1:50 with PBS for 1 hour; and (iv) FITC-conjugated streptavidin (Amersham) diluted to 1:100 with PBS for 1 hour.

PCNA was detected with an anti-PCNA human antiserum, and DNA was counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Sigma), or propidium iodide (PI; Sigma). The eggs, attached to a glass slide, were incubated according to the following order: (i) human antiserum against PCNA (a gift from Dr Yoshinari Takasaki; Suzuka et al., 1989) diluted to 1:100 with PBS for 2 hours; (ii) biotinylated sheep monoclonal antibody against mouse Ig (Amersham, Buckinghamshire, England) diluted to 1:50 with PBS for 1 hour; (iii) FITC-conjugated streptavidin (Amersham) diluted to 1:100 with PBS for 1 hour; and (iv) 0.2 µg/ml DAPI in PBS or 25 mg/ml PI in PBS for 1 hour. To use PI, eggs were preincubated in 2 mg/ml RNase A (Sigma) at 37°C to digest the endogenous RNA that is stainable with PI. When PBS was used for antibody dilution, it routinely contained 1% bovine serum albumin (Sigma).

In western blot experiments, a 36×10^3 Mr band, consistent with human PCNA (Mathews et al., 1984), was detected by the antiserum against PCNA. Here the molecules recognized by this antiserum will be tentatively regarded as ‘starfish PCNA.’

Finally, a drop of glycerol containing 10% 10mM Tris-HCl adjusted to pH 8.0 and 2.3% 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) was added to the eggs as an anti-fluorescence bleaching agent and covered with a coverslip. The specimens were examined using either a fluorescence microscope (Nikon Optiphot with EFD2, Tokyo, Japan) or a confocal laser scanning microscope (CLSM; MRC600, Bio-Rad, Tokyo, Japan) equipped to an Axioplan epifluorescence microscope (Carl Zeiss, Germany).

Fig. 1. Schematic illustration of temporal correlation between the maturation process and the postactivation process.

(A) Unfertilized eggs. Progression of the maturation process is represented by the white to gray gradation. The subsequent gray zone indicates that the maturation process has been completed. 'Egg' means a mature oocyte.

(B) Eggs fertilized during the maturation process. The progression of the postactivation process is illustrated by the inner rectangle with the gradient of white to dark gray. Since the postactivation process has been completed, the first S phase (1S) is initiated when the maturation process is completed. 2S, the second S phase. (C) Eggs fertilized after maturation. Since the maturation process has been completed, 1S is immediately initiated when the postactivation process is completed. GBVD, germinal vesicle breakdown; PB1 and 2, first and second polar body formation.
a personal computer (Macintosh Quadra 950, Apple Computer Inc., Tokyo, Japan). Several kinds of digital image processing, such as noise reduction and enhancement of the contrast, were made on the original images with either an application software (Photoshop 2.5, Adobe Systems Inc., Mountain View, CA) for digitally processing photographic images or a specific filtering software (Kai’s power tool 2.0, Harvard Systems Corp., Santa Monica, CA).

Analyzing the images obtained with CLSM

Optical sections of stained PCNA and chromatin were obtained with CLSM serially at a regular step of 0.48 \( \mu \text{m} \). Each image was digitized to numerical values (0-255) of 16×16 pixels for an area of 1 \( \mu \text{m} \times 1 \mu \text{m} \) on an optical section. The digitized images were stored in a storage device of a computer (AX/2, Nimbus, Research Machines Ltd, Oxford, UK) attached to the CLSM. The numerical data were exported to a Macintosh Quadra 950 using a data-conversion utility software (Access PC 2.1, Insignia Solutions Inc.). Several kinds of digital image processing were made on the digitized images with either a digital built-in filter within Photoshop or Kai’s power tool.

The digitized images of the serial section at 0.48 \( \mu \text{m} \) step are too far apart for three-dimensional reconstruction. Seven interpolated images were then computed for each pair of neighboring images, and inserted between them. This interpolation was done with an application software (Morph 2.0, Gryphon Software Corp., San Diego, CA) for transforming digital graphic images. As a result, a single pixel comes to represent the brightness of a cube of 1/16 \( \mu \text{m} \times 1/16 \mu \text{m} \times 0.48/8 \mu \text{m} \). All the digitized images including the interpolated images were integrated into a three-dimensional image with an application software (VoxelView/Mac 1.0, Vital Images, Inc., Fairfield, IA).

RESULTS

Timing the nuclear localization of PCNA

Starfish oocytes or eggs were fertilized either 40 minutes (early fertilized eggs) or 2 hours (late fertilized eggs) after 1-MA treatment. Their developmental events such as GVBD, polar body formation, and cleavages were determined visually. BrdU incorporated into the nuclei was detected with the anti-BrdU antibody. In this study, time of S phase is routinely defined as the time when more than 50% of eggs are labeled with BrdU. PCNA localized within the nuclei was also detected with the anti-PCNA antiserum. The percentages of PCNA-positive cells and BrdU-positive cells are shown in Figs 2 and 3.

In the early fertilized eggs, both the nuclear localization of PCNA and the first S phase were simultaneously initiated (Fig. 2A). In the late fertilized eggs, however, PCNA had already localized within the nuclei about 10 minutes before the initiation of the first S phase (Fig. 2B). The marked contrast in the time of PCNA localization between the two types of eggs will be referred to again. PCNA remained localized within the nuclei for 10-15 minutes after the end of the first S phase.

Localization of PCNA during the second and the third cell cycle was also examined (Fig. 3). In the early fertilized eggs, the behavior of PCNA was the same in each cell cycle as far as examined: it started to localize at the same time as the initiation of S phase, remained localized within the nuclei, and disappeared before the start of each metaphase (Fig. 3A). In the late fertilized eggs, PCNA had already localized within the nuclei.
when the first S phase was initiated. In the second and the third cell cycle, the behavior of PCNA was the same as in the early fertilized eggs: it started to localize at the same time as the initiation of S phase, remained localized within the nuclei, and disappeared before the start of each metaphase (Fig. 3B).

If not fertilized, formed pronuclei remain quiescent for hours without visible change. PCNA usually does not become localized to such pronuclei. Occasionally, however, I found some batches whose eggs were stained with anti-PCNA antiserum at the pronucleus. The batch shown in Fig. 3 is an example. Yet the time of the localization was widely scattered among eggs (Fig. 3C), and I have no explanation of such an unusual PCNA localization.

The distribution of localized PCNA and the chromatin structure

PCNA localized within nuclei was detected with the anti-PCNA antiserum and stained with FITC. Nuclear DNA was counterstained with DAPI. Fig. 4 illustrates a set of pictures of eggs, derived from the batch shown in Fig. 2, taken with a conventional fluorescence microscope. The first S phases are indicated by red frames surrounding the picture.

The results from the early fertilized eggs are shown in columns A (chromatin structure) and B (localized PCNA). Before the first S phase (one hour and 44 minutes after 1-MA treatment, cf. Fig. 2A), both egg and sperm nuclei had formed several small regions, indicating the formation of karyomeres.
Fig. 4. Localized PCNA within nuclei in the fertilized eggs of starfish. PCNA was detected by the anti-PCNA antiserum, and stained with FITC (columns B, D). DNA was also stained with DAPI (columns A, C). A series of images obtained from the early fertilized eggs are shown in columns A and B, and those obtained from the late fertilized eggs are shown in columns C and D. These two kinds of series were obtained from the same batch of eggs shown in Fig. 2. The numerals at the lower left corner of columns A and C indicate the time of fixation measured from 1-MA treatment (hour:minutes, for example 1:44 means the image obtained from eggs fixed at 1 hour and 44 minutes after 1-MA treatment). f, female chromatin; m, male chromatin. Bar, 10 µm. The red frames surrounding the pictures at 1:56 and 2:28 indicate S phase as detected by BrdU label (cf. Fig. 2).
during the first S phase, localized PCNA was detected in both female and male pronuclei (Fig. 4, 1:56). The distribution of localized PCNA appeared similar to that of chromatin. After the end of the S phase, PCNA remained localized, with the distribution nearly coincident with that of chromatin (Fig. 4, 2:04). When chromatin became condensed, PCNA was still detected but the distribution was limited within a portion of nuclei (Fig. 4, 2:12). When chromatin condensed further, localized PCNA was not detected (Fig. 4, 2:16). I have not clearly quantified the correlation between a condensation level of chromatin and the localization of PCNA.

The results from the late fertilized eggs are shown in column C (chromatin) and D (PCNA). In this type of eggs, as was previously reported, female pronuclei had been formed at fertilization (Nomura et al., 1991). Male pronuclei derived from the incorporated sperm nuclei were much smaller than the female pronuclei (Fig. 4, 2:12C). Soon after fertilization, localized PCNA was not yet detected either in the female or the male pronuclei (Fig. 4, 2:12 D). As already shown in Fig. 2B, about 10 minutes before the initiation of the first S phase, PCNA began to localize within the female pronuclei (Fig. 4, 2:24D). It should be noted that the distribution of localized PCNA was not coincident with chromatin that mainly distributed along the peripheral region of the nuclei (Fig. 4, 2:24C). The nuclear structure will be three-dimensionally examined later. During the first S phase (red frames), localized PCNA was detected in both female and male pronuclei (Fig. 4, 2:28D), and its distribution was coincident with chromatin (Fig. 4, 2:28C). After the end of the S phase, PCNA remained localized, with the distribution nearly coincident with that of chromatin. Even within condensing nuclei (Fig. 4, 2:56), PCNA was still detected, but the distribution of PCNA was limited within a portion of the nuclei. When chromatin condensed further, localized PCNA was not detected (Fig. 4, 3:08).

In both early and late fertilized eggs, during the second cell cycle, the distribution of localized PCNA was the same as that of the first cell cycle in the early fertilized eggs.

### Three-dimensional images of the PCNA region and the chromatin structure

Using a computer graphics program, a structure where localized PCNA was distributed was three-dimensionally imaged on the computer display. Localized PCNA was detected with the anti-PCNA antiserum and stained with FITC. Nuclear DNA was counterstained with PI instead of DAPI, since the CLSM used in this study did not detect the DAPI signal. In my illustration of the reconstructed structure, PCNA was shown in green color, and the chromatin was shown in red. At first, images of the whole nuclei were reconstructed by simply stacking the optical serial sections, and the outermost surfaces of the nuclei were illustrated. To visualize the inner part of the nuclear structure, two kinds of modifications were made. One is dividing the reconstructed structure into several partitions along an axis. They were illustrated as they were separated from each other. These types of image will be called ‘partitioned images.’ The other modification is to make transparent the green area of localized PCNA. By this modification, even the chromatin structure lying behind the green area became partially visible through it. These types of image will be called ‘transparent images.’ Images of both PCNA region (green) and chromatin structure (red) were merged. Thus, the area of chromatin where PCNA was co-distributed appears yellow. Figs 5-9 show the results obtained from the batch shown in Fig. 2.

A reconstructed image of the male and female pronuclei during the first S phase in the early fertilized eggs is shown in Fig. 5A, and its four partitioned images are shown in A1-A4. At this stage, the distribution of localized PCNA extended to the whole pronuclei, and entirely coincided with the chromatin distribution, as shown by a predominant yellow area in the partitioned images (Fig. 5, A1-A4). After the end of the first S phase, localized PCNA was distributed only within a certain portion of the nuclei (Fig. 6A,B). Most PCNA was not co-distributed with the chromatin. Some chromatin assumed a vesicular appearance, probably representing forming chromosomes. Some of them were wrapped around a zone of the PCNA region (Fig. 6, B1-B4). This is well illustrated by the mosaic pattern of the green, red, and yellow areas in the partitioned images (Fig. 6, A1-A4).

Nomura et al. (1993) reported that the chromatin of the female pronuclei in the late fertilized eggs showed a hollow structure. This hollow structure was also observed in the late fertilized eggs examined in this study. Chromatin was distrib-
Nuclear distribution of PCNA in starfish eggs

uted mainly at the periphery and was sparsely distributed at the central region of the nuclei (Fig. 7, A1-A4).

Before the first S phase (Fig. 7), localized PCNA (green) clustered on one side of the inner space of the nuclei without diffusing over the whole nuclear area. A part of the chromatin was found within the PCNA region (Fig. 7, A1-A4). This is well illustrated by the mosaic pattern of the partitioned images (Fig. 7, A1-A4). When the first S phase was initiated (Fig. 8), the mosaic pattern disappeared and the yellow area extended widely in the partitioned images (Fig. 8, A1-A4). After the end of the first S phase, the three-dimensional structure of the nuclei was quite similar to that of the same stage in the early fertilized eggs shown in Fig. 6. Localized PCNA was distributed only within a certain portion of the nuclei (Fig. 9A,B). Most PCNA was not co-distributed with the chromatin. Some vesicular chromatin was wrapped around a zone of the PCNA region (Fig. 9, B1-B4). This is well illustrated by the mosaic pattern in the partitioned images (Fig. 9, A1-A4).

Going back to Figs 7 and 8, I notice that the clustering of PCNA distribution (green + yellow) on one side of the nuclei seen before the first S phase (Fig. 7) persists through the S phase (Fig. 8). This persistence of the clustering PCNA will be referred to in the Discussion. On the other hand, the chromatin (red + yellow) distributes around the peripheral area of the nuclei mostly outside the PCNA region before the S phase (Fig. 7), and then becomes coincident with the PCNA region with the initiation of the S phase (Fig. 8).

**DISCUSSION**

**Use of the anti-PCNA human antibody for detecting the nuclear localization of starfish PCNA**

To detect a starfish equivalent of PCNA, the human anti-PCNA antiserum was used. This antiserum is known to recognize PCNA of various species, from higher plant PCNA to mammal
PCNA (Suzuka et al., 1989). In the present study, the molecule in starfish eggs recognized by this antiserum was tentatively called ‘starfish PCNA,’ on the basis of western blot analysis. I found that the molecules recognized by the antiserum become localized to chromatin in S phase nuclei, as do genuine human PCNA molecules. This strongly indicates that the molecules have such a function in DNA replication as to be properly called ‘starfish PCNA.’

In DNA replication, PCNA molecules firmly bind to the replication fork and are not easily extractable (see van der Vliet, 1989; Laskey et al., 1989, for review). Based on results obtained by Bravo and McDonald-Bravo (1987), localized PCNA detected in this study is considered to represent those that tightly bind to nuclei and are not extracted from nuclei with the methanol fixation.

It should be a very interesting subject to relate the distribution of localized PCNA with that of DNA replication sites, by simultaneous detection of PCNA and BrdU. Working preliminarily on starfish eggs, I realized, however, that the signal of localized PCNA is often weakened after HCl treatment, a procedure essential for the anti-BrdU antibody to recognize incorporated BrdU. In a foregoing paper (Nomura et al., 1993), I have already confirmed that the distribution of DNA replication sites detected by a BrdU pulse labeling was identical to the chromatin distribution during the first and second S phases of starfish eggs. Based on this observation, the chromatin distribution was taken to represent the sites of DNA replication in this paper. Efforts were recently made to overcome the difficulty in simultaneously detecting DNA replication sites and PCNA: Kill et al. (1991) used biotin-11-dUTP to label the DNA replication sites, and Humbert et al. (1992) adopted an enzymic procedure for denaturing the DNA instead of chemical denaturation. I have not yet established if these methods apply to starfish eggs.
Temporal correlation between the nuclear localization of PCNA and the first S phase

A delay between the nuclear localization of PCNA and the initiation of DNA replication has been noted (Celis and Celis, 1985; Hutchison and Kill, 1989; Nakane et al., 1989). The techniques improved by Kill et al. (1991) and Humbert et al. (1992) have led them to confirm the presence of such a delay. Kill et al. (1991) observed the delay in human diploid fibroblasts cells and Xenopus eggs, and proposed the idea that the entry into S phase is biphasic; assembly of the replication complex including PCNA is followed by a process in which those complexes are used. Humbert et al. (1992) also observed the delay and ascribed it to a time-lapse between the assembly of replication complexes and the initiation of DNA replication. Most recently, Baptist et al. (1993) proposed an unknown process ‘X,’ programed after the nuclear localization of PCNA, with a function necessary for DNA replication.

As I found in the late fertilized eggs of starfish, the precocious localization of PCNA within female pronuclei about 10 minutes before the initiation of the first S phase appears to generalize those delays mentioned above. It should be noted, however, that the occurrence of the delay depends on the time of fertilization in starfish: in the early fertilized eggs, PCNA started to localize at the same time as the initiation of the first S phase. What is unique in starfish eggs is that the time of fertilization alone makes such a contrast in PCNA localization.

Nomura et al. (1991, 1993) proposed the term ‘postactivation process’ to imply a cascade preparing for DNA replication in starfish eggs. The delay observed in the late fertilized eggs indicates that the postactivation process induces the nuclear localization of PCNA at 20 minutes after fertilization and DNA replication at 30 minutes. Thus, localized PCNA turns out to be a marker midway in the progress of the postactivation process. In the early fertilized eggs, both nuclear localization of PCNA and initiation of the first S phase immediately followed the completion of the maturation process with the formation of male and female pronuclei. These results support the idea that the postactivation process must be going on and must complete in parallel with the maturation process.

Spatial correlation between the distribution of PCNA and the chromatin structure

In addition to the delay between the nuclear localization of PCNA and the initiation of DNA replication, Kill et al. (1991) observed that the preceding distribution pattern of PCNA often resembles the expected distribution pattern of DNA replication sites. Humbert et al. (1992) reported that a few of the PCNA-positive/BrdU-negative cells show a PCNA distribution very similar to the distribution at an early S phase.

Present studies on the late fertilized eggs of starfish confirmed such a similarity, or a persistence of the PCNA distribution as a cluster on one side of the nucleus before and during S phase. By merging PCNA and DNA label in the three-dimensionally reconstructed images on the computer display, I further observed that the precocious distribution of PCNA is not identical to the chromatin distribution. This implies that most PCNA molecules precociously localized within female pronuclei cannot contact with the chromatin before the first S phase. When the S phase is initiated, the distribution of PCNA and chromatin become coincident with each other.

The persistence of the PCNA cluster before and during the first S phase in turn will imply a dynamic relocation of the chromatin mass towards the PCNA cluster for the onset of DNA replication. Thus, S phase is correlated with a redistribution of chromatin to the PCNA sites in the nucleus. It appears that such a relocation of chromatin mass has been unnoticed heretofore, but the three-dimensional image analysis made in the present study shows that it actually the case at the start of the first S phase at least in the late fertilized eggs of starfish. If PCNA distribution can be looked upon as sites of functional replication complexes, then my observation suggests that replication complexes are formed not strictly at replication forks but as discrete sites in the nucleus that then become future sites of replication.

In the early fertilized eggs and the second cell cycle of the late fertilized eggs, the precocious localization of PCNA was absent, and the distribution of PCNA was coincident with the chromatin distribution at the start of the localization. This suggests that PCNA properly reaches the DNA replication fork as soon as the nuclei has been formed. The direct localization of PCNA into the DNA replication fork should result in the rapid start of DNA replication, which will help to shorten the cell cycle periods in early development.

A built-in stability of the cell-cycle in starfish embryos

No matter how the S phase initiation has a delay behind the nuclear localization of PCNA, the disappearance of PCNA was always scheduled at the initiation of chromatin condensation (cf. Fig. 3). The second and the third cell cycle was quite regular irrespective of the time of fertilization. In a previous paper, Nomura et al. (1993) suggested that ‘the cell cycle driving mechanism that is perturbed by the interaction of the maturational process with the postactivation process could be stabilized when the first round of the cell cycle is completed’. Timely disappearance of localized PCNA from nuclei of the starfish eggs as observed in the present study provides further evidence for the operation of a certain device to ultimately stabilize the cell cycle driving mechanism. As discussed earlier (Nomura et al., 1993), this built-in stability of the cell-cycle driving mechanism will be an essential device for normal development of starfish eggs that are naturally fertilizable at any time of maturation without the ‘metaphase arrest’ known in most animal eggs.

I thank Dr Mitsuki Yoneda for reading the manuscript, and giving helpful discussions. The western blotting experiment was done by Mr Tatsuya Ueno of Kyoto University. I also thank Dr Yoshinari Takasaki in Juntendo University for giving me the anti PCNA-antiserum. I express my gratitude to Dr Shoji Tanaka in Mitsubishi Kasei Institute of Life Sciences for instructing me to use the CLSM at the institute and encouraging me to complete this work. Most of the work was performed at Tateyama Marine Laboratory, Ochanomizu University, Umi-no-Hoshi, Tateyama, Chiba. I am grateful to Dr Sin-ichi Nemoto, the director and Mr Mamoru Yamaguchi and Mrs Hisae Kikuchi, staff of the laboratory for providing me with the facilities for research. I also thank Mr Tamio Arano in the ‘Zero-One Shop, Cannon (Esaka branch)’ for his technical support to my personal computer. I am grateful to Mr Yoshimi Miyata, Mr Shin-ichi Kajiya and other members in Risuken for giving me an opportunity to master the computer programing. This work was supported in part by a grant-in-aid for JSPS fellows.
REFERENCES


(Received 6 June 1994 - Accepted 16 August 1994)