DNA synthesis after polyspermic fertilization in the axolotl

By B. T. WAKIMOTO

From the Department of Biology, Indiana University

SUMMARY

Cytological and autoradiographic studies were done to investigate the cytoplasmic control of DNA synthesis under conditions of physiological polyspermy. The DNA synthetic phases of the egg, principal sperm and accessory sperm nuclei were determined and correlated with nuclear morphology and developmental fate. Results show that accessory sperm nuclei undergo morphological transition to pronuclei. Their DNA synthetic phase is the same as that of the principal sperm nucleus. Hence accessory sperm nuclei are capable of initiating and completing DNA replication before any cytological evidence of their degeneration is observed.

INTRODUCTION

The eggs of urodele amphibians are normally polyspermic. Up to ten or more sperm may enter a single egg. Although all sperm transform into pronuclei and develop the sperm aster, only one fuses with the egg pronucleus. After this occurs, supernumerary sperm begin to degenerate and play no discernible role in development (Fankhauser, 1932, 1948). The mechanism whereby the supernumerary sperm are suppressed while the fusion nucleus in the same cytoplasm is unaffected remains unknown. One aspect of this problem, that of DNA synthesis in the principal sperm nucleus, the accessory sperm nuclei and the female pronucleus, is examined in this report.

In monospermic eggs it is known that DNA synthesis is activated shortly after fertilization. Graham (1966), Graham, Arms & Gurdon (1966) and Gurdon (1967) have shown that if brain nuclei or extra sperm nuclei are injected into *Xenopus* eggs, they too initiate DNA synthesis. However, these monospermic eggs lack a mechanism for suppressing the extra sperm nuclei. Although the accessory sperm undergo DNA synthesis, they also contribute extra cleavage centers. The result is the formation of multipolar spindles and aneuploidy. Normal development is invariably disrupted. In the case of normally polyspermic eggs, the extra sperm nuclei are suppressed. It is not known whether this also involves a suppression of DNA synthesis, or an altered pattern of the supernumerary nuclei relative to that of the principal sperm or egg nucleus.

1 Author's address: Department of Biology, Indiana University, Bloomington, Indiana, 47401, U.S.A.
In the present work, this question has been examined in axolotl eggs. The DNA synthetic phases of the principal sperm nucleus, the accessory sperm nuclei and the egg nucleus were determined autoradiographically. These phases were correlated with morphological changes seen in each type of nucleus from the period between insemination and the first cleavage division.

**MATERIALS AND METHODS**

**Egg collection**

Fertilized eggs were collected at 15 min intervals from spawning female axolotls or were obtained by artificial insemination. The eggs were placed in 10% sterile Steinberg’s saline (Steinberg, 1957) in a waterbath maintained at 18 ° ± 0.05 °C. After 15 min the surfaces of the eggs were observed for the presence of the crater-like sperm pits. Those showing two or more sperm pits were selected and the number and distribution of sperm pits were recorded for each polyspermic egg. Eggs were then returned to the constant temperature waterbath until injections were made.

**Labeling Methods**

[3H]Thymidine ([3H]Thy) was used as a specific label for DNA synthesis. [3H]Thy labeled at the 6C position, was obtained from Radiochemical Centre, Amersham, England. The original concentration was 26 Ci/mmmole. Fifty nl of a 1 mCi/ml solution of [3H]Thy in sterile full strength Steinberg’s saline was microinjected into eggs at various times after fertilization. All injections were performed with micropipettes 10–15 μm in diameter attached to a Leitz micromanipulator. The total concentration of radioactivity inside the egg was approximately 12 μCi/ml. After injection, the egg was then incubated at 18 ± 0.05 °C for 1 h in Steinberg’s saline containing twice the standard concentration of Ca²⁺ and Mg²⁺ to facilitate healing.

**Preparation of autoradiographs**

After incubation, the eggs were fixed in Masui’s fixative (Ziegler & Masui, 1973), embedded in paraffin wax, then serially sectioned at 7 μm. The sections were placed in a solution of 10% H₂O₂ in 63% ethanol for 20 h to bleach pigment granules (Moore, 1963). Hydrolysis in 1 N-HCl was carried out at 60 °C for 11 min before staining in Feulgen reagent and counterstaining in Fast Green. Slides were dipped in Kodak Nuclear Track Emulsion Type NTB2 and exposed for two weeks at 4 °C. After the autoradiographs were developed, the sections were restained with Fast Green and scored for the incorporation of [3H]thymidine.
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Fig. 1. Summary of nuclear events and DNA synthetic activity of fertilized axolotl eggs. Development is at 18 °C; time is in hours after insemination. The solid regions represent the periods during which the various nuclei incorporate [3H]thymidine. Numbers in parentheses refer to the number of eggs observed within each time period. These served as the basis for the cytological descriptions.

RESULTS

The results of cytological and autoradiographic studies are summarized by Fig. 1. Cytological data were obtained from spawnings of eight different axolotl females. Eggs from five of these females were injected with [3H]thymidine for autoradiography. Variation among individual females with respect to cytological events was ± 30 min of the time scale depicted in Fig. 1. Table 1 indicates a variability of ± 15 min for the time of initiation and completion of DNA synthesis as resolved by autoradiography. Timing was begun (T = 0) when the sperm suspension was added to the eggs during artificial insemination or the time of egg collection from natural spawnings.

Cytology of polyspermic fertilization

The cytology of polyspermic fertilization in the axolotl was found to be very similar to that described for Triturus viridescens by Fankhauser & Moore (1941). Fig. 1 summarizes the timing of nuclear events for the axolotl. The descriptions are based on a total of 98 eggs. Fig. 2 illustrates the changes observed in the egg and sperm nuclei leading to the formation of the zygote nucleus and the completion of the first cleavage mitosis by 7 h after
Table 1. The incorporation of [\textsuperscript{3}H]thymidine after polyspermic fertilization

<table>
<thead>
<tr>
<th>Labeling interval\textsuperscript{*}</th>
<th>1–2</th>
<th>2–3</th>
<th>3–4</th>
<th>4–5</th>
<th>5–6</th>
<th>6–7</th>
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<tr>
<td></td>
<td>$\frac{1}{2}$–1 $\frac{1}{2}$</td>
<td>1–2 $\frac{1}{2}$</td>
<td>2$\frac{1}{2}$–3 $\frac{1}{2}$</td>
<td>3$\frac{1}{2}$–4 $\frac{1}{2}$</td>
<td>4$\frac{1}{2}$–5 $\frac{1}{2}$</td>
<td>5$\frac{1}{2}$–6 $\frac{1}{2}$</td>
</tr>
<tr>
<td>No. of eggs...</td>
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<td>5</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Egg nuclei</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. observed</td>
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<td>5</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>No. labeled</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Zygote nuclei</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. observed</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>No. labeled</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Sperm nuclei\textsuperscript{†}</td>
<td>12</td>
<td>14</td>
<td>20</td>
<td>7</td>
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<td>8</td>
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<td>0</td>
<td>0</td>
<td>6</td>
<td>7</td>
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</table>

* Incubation periods were 1 h in duration; intervals are expressed as hours after insemination.
† Number of sperm nuclei exclusive of principal sperm nucleus after formation of the fusion nucleus.

insemination. Fig. 3 shows the cytological changes leading to the degeneration of the accessory sperm nuclei.

Sperm penetrate the axolotl egg at any point along its surface. Irrespective of their location within the egg, the accessory sperm undergo the same morphological transition from condensed sperm heads to swollen pronuclei. However, sperm located in the yolky vegetal hemisphere may lag as much as 30 min behind those of the animal or equatorial regions of the same egg.

Accessory sperm in the upper half of the egg are indistinguishable from the principal sperm nucleus for the first three hours after insemination. The accessory sperm show pronuclear morphology and continue to swell until 5 h

**Figure 2**

The cytology of polyspermic fertilization in the axolotl. Magnifications for Fig. 2A–G are the same.

(A) At approximately 1–1 $\frac{1}{2}$ h after insemination, the second meiotic division is completed with the release of the second polar body (\textit{pb}). The egg nucleus (\textit{en}) lies at the uppermost animal pole.

(B) At 3 h post-insemination, the egg nucleus had moved deeper into the egg and shows a swollen pronuclear morphology.

(C) The sperm head at 1 $\frac{1}{2}$ h after insemination appears condensed and elongated.

(D) By 2 h the sperm nucleus has decondensed slightly and appears as a pear-shaped intermediate.

(E) The sperm nucleus becomes more rounded by 3 h. It often carries pigment granules into the egg along its penetration path as shown here.

(F) The sperm nucleus shows the swollen pronuclear morphology by 4 h after insemination. It is associated with a darker staining centrosome (\textit{c}).

(G) The male (upper) and female (lower) pronuclei are in contact at 4 h.

(H) By approximately 6 h the fusion nucleus has undergone chromosome condensation and is at metaphase of the first cleavage mitosis.
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Fig. 3. Accessory sperm nuclei. (A) Accessory sperm nucleus at 5 h after insemination appears lightly stained. The centrosome (c) is clearly visible. This is an autoradiograph showing light labeling of the accessory sperm nucleus after an incubation period from 4 h to 5 h after insemination. (B)-(E) Degenerating accessory sperm nuclei at $5\frac{1}{2}$–6 h. Intense chromatin condensation has begun and is often localized to the region near the centrosome (c) as shown in Fig. 5C. The nuclei of Fig. 5C, D and E are from the same egg. The zygote nucleus of this egg was in metaphase of the first cleavage mitosis.

(Fig. 3A). When Feulgen positive chromosomes are first seen in the zygote nucleus, the accessory sperm nuclei also begin chromatin condensation. By $5\frac{1}{2}$–6 h chromatin condensation has become intense and nuclei are irregularly shaped. These changes lead to highly variable degenerative changes and pyknosis of the accessory nuclei (Fig. 3B–E).
Fig. 4. DNA synthesis after polyspermic fertilization. (A) Autoradiograph showing intense labeling of the female pronucleus from an egg injected with label at 2½ h and incubated until 3½ h after insemination. (B) Autoradiograph of the male pronucleus from an egg with the same labeling interval as (A). (C) Autoradiograph of a fusion nucleus showing equal intensities of labeling of the female (left) and male (right) halves. The labeling interval was from 2½ to 3½ h. Because the fusing pronuclei had different planes of focus, this montage was created to present the grains in focus over both halves of the fusion nucleus. (D) Autoradiograph of a fusion nucleus labeled from 3½–4½ h. The striking difference in grain intensities of the male (left) and female (right) halves is indicative of asynchronous DNA synthesis. (E) and (F) The accessory sperm nuclei from the vegetal hemisphere of a single egg. The labeling interval was from 3–4 h. The fusion nucleus of this egg was also heavily labeled.
DNA synthesis in polyspermic eggs

[3H]thymidine was injected into eggs at times ranging from 30 min to 6 h after insemination. This was followed by 1 h incubation periods. The following data are based on 54 eggs serially sectioned and prepared for autoradiography.

The egg and sperm nuclei are first seen to incorporate [3H]thymidine at 2½—3 h after insemination (Table 1). DNA synthesis begins prior to pronuclear fusion but after the nuclei have shown a morphological transition to swollen pronuclei (Fig. 4A, B). Sperm nuclei within the same cytoplasmic region appear identical in morphology and in intensity of labeling. Maximum incorporation is seen at 3½ h in sperm nuclei and synthesis is completed at 4 h. The synthetic period for the egg nucleus lasts no longer than 4½ h. No labeling was ever found in eggs injected after this time. These results are summarized by Fig. 1.

Over half (9/16) of the autoradiographs of pronuclei in contact show differential labeling of male and female halves. A striking example of this is shown in Fig. 4D. The incubation period of this egg (from 3½ to 4½ h) was near the termination of the DNA synthetic period. Only the female half continues the uptake of [3H]thymidine. The results indicate that male and female pronuclei replicate their DNA asynchronously. The female pronucleus completes DNA synthesis later than the male pronucleus.

DISCUSSION

Cytological observations show that the timing of nuclear events and the behavior of degenerating sperm nuclei in the axolotl closely resemble those of Triturus viridescens (Fankhauser & Moore, 1941). Autoradiographs show that DNA synthesis in the axolotl begins independently in the pronuclei and prior to pronuclear fusion. This is similar to DNA synthesis after fertilization in Xenopus (Graham, 1966).

Slight asynchrony exists between the male and female pronuclei in the axolotl. These experiments were unable to detect a difference in the time at which the male and female pronuclei initiate DNA synthesis. However, a difference was seen for the time of termination of the DNA synthetic period. The unequal intensities of labeling by the two pronuclei in contact further substantiate that these nuclei are asynchronous in DNA synthesis. In the majority of species studied (Johnson & Rao, 1971), the male and female pronuclei are found to begin and end DNA synthesis simultaneously. However, Sirlin & Edwards (1959) and Luthardt & Donahue (1973) reported that DNA synthesis occurs earlier in the male compared to the female pronucleus of the mouse. In both the mouse and the axolotl, the interval from insemination to the first cleavage division is quite long (11 h and 7 h, respectively) compared to that of other species studied (e.g. 70 min in Xenopus). The longer interval may allow any existing asynchrony to be detected. Since the egg and sperm differ markedly in
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nuclear state at the time of insemination, it is not surprising to find that these nuclei require different times to undergo necessary nuclear changes for DNA synthesis.

The results presented here show that the cytoplasm of a physiologically polyspermic egg initiates DNA synthesis in accessory sperm nuclei. The initial response of accessory sperm nuclei in these eggs is the same as in normally monospermic eggs. In both cases, morphological changes and pronounced nuclear swelling precede the initiation of DNA synthesis. The DNA synthetic phase of the accessory sperm nuclei is within the same time period as that of the principal sperm. However, an additional nucleocytoplasmic interaction is present in urodele eggs that subsequently initiates the suppression of accessory sperm nuclei. Although the mechanism responsible for suppression of accessory sperm nuclei in these eggs is not yet known, it is clear that the inability of a sperm nucleus to fuse with the egg nucleus is not correlated with its failure to undergo DNA synthesis. Indeed, the DNA synthetic phase appears to be completed before any cytological evidence of degeneration begins in the supernumerary sperm.

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REFERENCES


The activity of maturing oocytes inducing chromosome condensation in transplanted brain

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