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Production of Diploid Eggs through Premeiotic Endomitosis in the Hybrid Medaka between *Oryzias latipes* and *O. curvinotus*

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ABSTRACT—A hybrid medaka between *Oryzias latipes* and *O. curvinotus* spawns diploid eggs. We examined the cytological mechanisms of diploid egg formation in this hybrid. Oocytes in the hybrid passed through the first and second meiotic divisions and excluded two polar bodies, associated with reduction of the DNA content in oocytes. Each germinal vesicle in vitellogenic oocytes of the hybrid had 48 chromosomes with bivalent chiasmata, precisely twice the number of chromosomes in normal oocytes. These results suggest that before meiosis the chromosomes are doubled by mitosis without cytokinesis, that is, endomitosis, and that the resulting tetraploid oogonia undergo normal meiosis to produce diploid eggs. Except for a few vitellogenic oocytes that are probably derived from endomitotic oogonia, most oocytes were arrested at the zygotene stage in the hybrid ovary, suggesting the existence of checkpoint control that ensures the pairing of homologous chromosomes at prophase I, a situation differing from that in the male in which the checkpoint is at metaphase I. A sac-like structure was characteristic of the hybrid ovary. Although this structure was observed only in the hybrid, it may be a native component of the medaka ovary but difficult to observe because of its deformed outer structure due to enlargement of the inside oocytes. The present study confirms that this hybrid medaka provides a useful experimental system for investigations into the mechanisms of oogenesis and basic architecture of the ovary, which are difficult to analyze by using normal medaka.

INTRODUCTION

Gametogenesis is a complex process that includes proliferation of germ cells, progression of meiotic cell division, and transformation into gametes (the egg and spermatozoon). Although spermatogenesis and oogenesis are cytologically identical in terms of meiosis, they differ in various aspects. For example, oocytes are arrested once at the diplotene stage of meiotic prophase I, during which substances (ex., mRNAs and yolk proteins) necessary for early embryonic development are accumulated (this stage being called vitellogenesis), whereas spermatocytes do not undergo such an arrest during meiosis. Critical checkpoint control must operate on the arrest of growing oocytes at the diplotene stage, but the entire mechanism still remains unclear in vertebrates.

Hybrids between closely related species often develop normally but become sterile or sometimes produce abnormal gametes. Like genetic mutants that are extensively used for analyzing other complex biological processes, appropriate hybrids are expected to provide a powerful tool for investigating the control mechanisms of gametogenesis, which are difficult to investigate by using normal organisms. In fact, 5 hybrid sterility loci have been identified using F_1 hybrids between mouse species (Forejt, 1996). Among lower vertebrates, the medaka (genus *Oryzias*) is a useful model animal (Naruse *et al*., 1994), and the possibility of producing F_1 hybrids from closely related species among the genus *Oryzias* has already been examined extensively (Iwamatsu *et al*., 1984, 1986, 1994; Sakaizumi, 1985, 1986; Uwa, 1986; Uwa and Parenti, 1988; Sakaizumi *et al*., 1992, 1993; Kurita *et al*., 1995). It is highly likely that we can gain an insight into the regulatory mechanisms of gametogenesis by investigating the cause of hybrid-specific abnormalities in the medaka.

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The F_1 hybrid between *O. latipes* and *O. curvinotus* exhibits sex-specific abnormalities; the male is sterile and the female lays diploid eggs (Hamaguchi and Sakaizumi, 1992; Sakaizumi *et al.*, 1992, 1993). We have previously examined the process of spermatogenesis in this hybrid and found that meiosis stops just before metaphase I, probably due to incomplete chromosomal pairing (Shimizu *et al.*, 1997). Nevertheless, spermiogenesis proceeds and sperm-like cells each having tetraploid (4C) DNA are produced without prior meiosis (Shimizu *et al.*, 1997). The mature sperm-like cell has a large head and does not swim, causing male sterility (Hamaguchi and Sakaizumi, 1992).

Following the analyses of spermatogenesis in the hybrid medaka between *O. latipes* and *O. curvinotus*, we examined the cytological mechanisms of diploid egg formation in this hybrid. Histological observations and DNA fluorometry indicated that diploid eggs are produced through normal meiosis that follows an extra DNA replication by endomitosis occurring once in oogonia just prior to the premeiotic DNA replication.

MATERIALS AND METHODS

Hybrid fish

Oryzias latipes from Suwa City, Nagano Prefecture, Japan and *O. curvinotus* from Hong Kong raised at Shinshu University were crossbred to generate inter-specific hybrids as described previously (Shimizu *et al.*, 1997). The hybrid and parent medakas were cultured under reproductive conditions (14-hr light and 10-hr dark at 28°C).

Histological observation and DNA quantitation

Ovaries isolated from mature females were fixed in Bouin's solution, embedded in paraffin, serially sectioned at 5 μm, and stained with Carrazzi's hematoxylin and eosin to examine oogenesis histologically. Changes in morphology of spindles and chromosomes during meiosis were examined as follows (Iwamatsu, 1997). Ovaries were surgically removed from the fish 3 hr before light onset. Maturing oocytes that had undergone germinal vesicle breakdown were isolated from the ovary and cultured in medaka Ringer's solution (Iwamatsu, 1973) at room temperature. According to the schedule of meiosis (Iwamatsu, 1965; Yamauchi and Yamamoto, 1973), oocytes were fixed in medaka Ringer's solution containing 4% glutaraldehyde for 4 hr at 4°C for examining meiotic metaphase I, anaphase I, metaphase II and anaphase II. After fixation, the cortical cytoplasm at the animal pole was skinned from the underlying yolk mass using a scalpel in Ringer's solution and embedded in 1% agar. The agar blocks were embedded in paraffin, serially sectioned at 5 μm, and stained with Carrazzi's hematoxylin and eosin. Diameters of spindles at metaphases I and II were measured under a light microscope. For measuring DNA contents in oocytes, pieces of the cortical cytoplasm at the animal pole were placed on glass slides, stained with 1 μg/ml Hoechst 33258 dye in distilled water for 30 min at room temperature, and observed using a fluorescent microscope. Fluorescence intensity from Hoechst 33258 was measured using a P1 fluorometer (Nikon, Tokyo).

For observing chromosomes in vitellogenic oocytes, germinal vesicles (18 from 3 individuals of *O. latipes* and 54 from 15 individuals of the hybrid medaka) were isolated from the oocytes with forceps in Ringer's solution. After being washed by gentle pipetting, they were transferred into 1 μg/ml Hoechst 33258 dye on glass slides and examined under a fluorescence microscope.

RESULTS

Ovarian structure and oogenesis in the hybrid medaka

The ovarian cavity at the dorsal side of the hybrid ovary was well developed, as it is in the parent species (Fig. 1). The ovaries of the parent species contained many large, growing oocytes at the diplotene stage (Fig. 1A). In contrast, the ovary of the reciprocal hybrid had only a few growing oocytes at

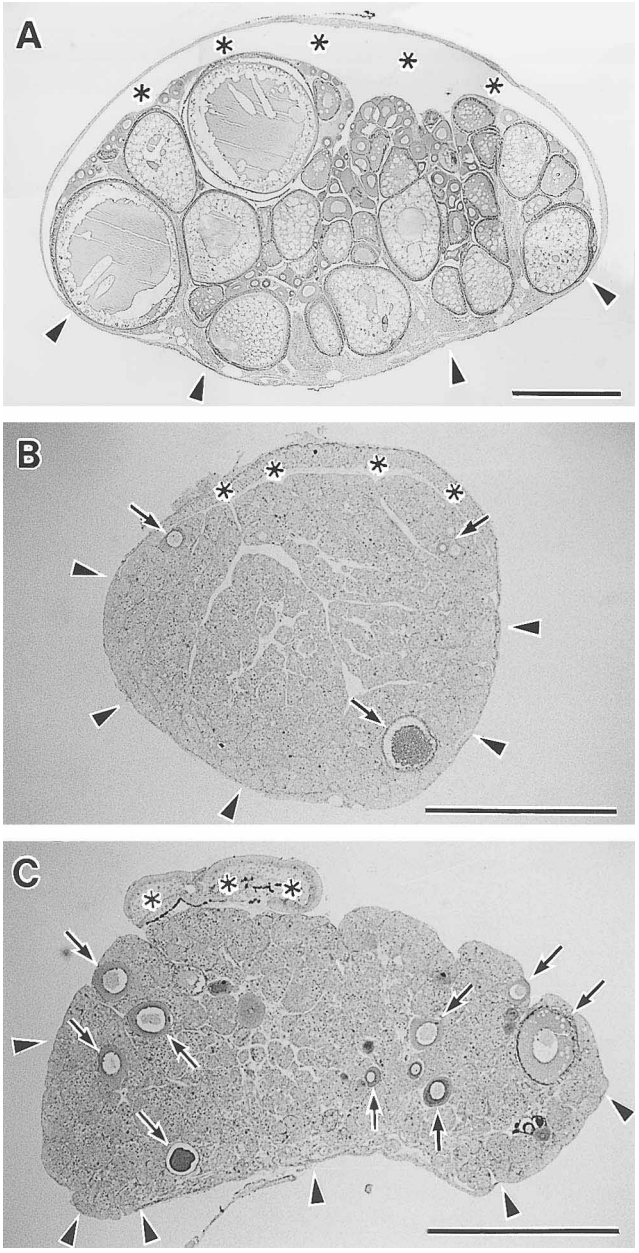

Fig. 1. Hematoxylin-eosin (HE)-stained histological sections of ovaries from *Oryzias latipes* (A) and reciprocal hybrids, female *O. latipes* x male *O. curvinotus* (*O. latipes-curvinotus*, B) and female *O. curvinotus* x male *O. latipes* (*O. curvinotus-latipes*, C). Asterisks show the ovarian cavity, arrowheads show ovarian stroma, and arrows show growing oocytes at the diplotene stage in the hybrid medaka ovary. The ovarian cavity of *O. curvinotus-latipes* was damaged upon removal. Bar = 500 μm.

diplotene stage (Figs. 1B and C). In the hybrid ovary, we also found many sac-like components each consisting of a single layer of flattened somatic cells (Figs. 2 and 3B), which were not seen in the normal ovary (Fig. 3A). In the parent species, oogenesis progressed synchronously until the pachytene stage, where the oocytes clustered together, and supporting cells around the oocytes become follicle cells at the diplotene stage (Fig. 3A). In the hybrid, oogonia and zygotene oocytes were dominant in the sac-like structure (Fig. 3B). Somatic cells were also observed in the sac-like structure (Fig. 3B). A small number of diplotene oocytes found in the hybrid ovary existed mostly as single cells of various sizes (Figs. 1B and C), in striking contrast to those found in the parent species, which constituted groups in the ovary (data not shown).

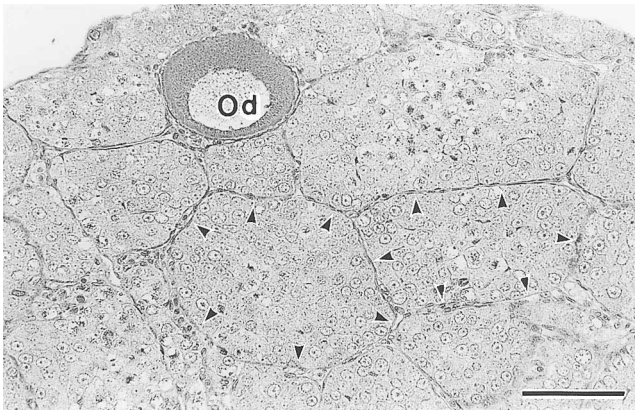

Fig. 2. An HE-stained section showing the sac-like structure in the hybrid medaka. The margins of the sac-like structure are indicated by arrowheads. The sac-like structure consists of a single layer of somatic cells. Growing oocytes at the diplotene stage (Od) are surrounded by follicle cells emerging from the sac-like structure. Bar=50 μm.

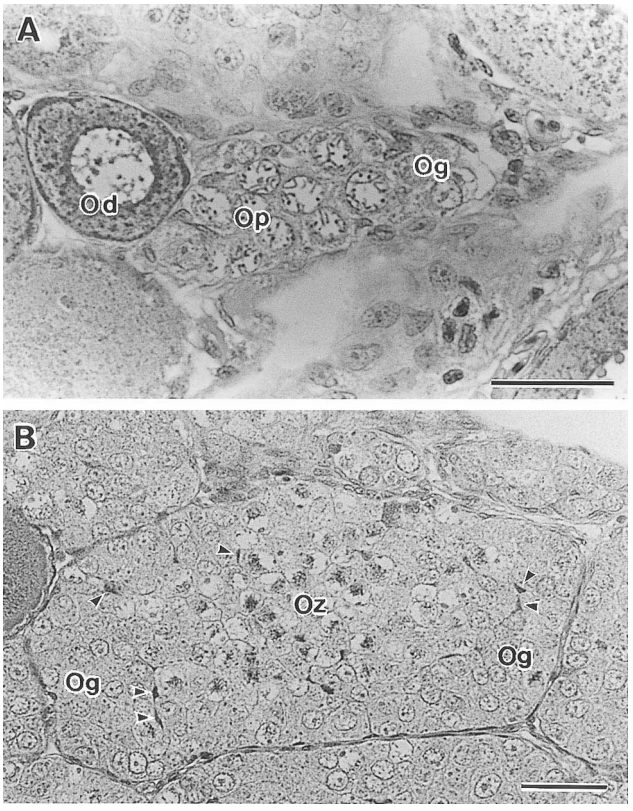

Fig. 3. HE-stained sections showing germ cells in the normal (A) and the hybrid (B) medaka. In the ovary of the normal medaka (A), diplotene oocytes (Od) surrounded by follicle cells, pachytene oocytes (Op) clustered together, and premeiotic oogonia (Og) can be seen. In the hybrid medaka (B), most of oocytes fail to enter the pachytene stage, thereby remaining at the zygotene stage (Oz) in the sac-like structure. Numerous oogonia (Og) and some somatic cells (arrowheads) are also present in the sac-like structures. Bar = 25 μm.

Changes in chromosomes during meiosis in the hybrid medaka

In *O. latipes* oocytes, chromosomes were aligned at the first metaphase plate 2 hr before ovulation (Fig. 4A), and the first polar body was excluded 1 hr before ovulation (Fig. 4C). At the time of ovulation, chromosomes were aligned at the second metaphase plate (Fig. 4E), and by 15 min after insemination the second polar body was excluded (Fig. 4G). The observed time course of chromosome alignment and polar body exclusion was consistent with that described previously (Iwamatsu, 1965; Yamauchi and Yamamoto, 1973).

In the hybrid oocytes, the alignment of chromosomes at the first and second metaphase plates and the exclusion of the first and second polar bodies occurred on the same schedule as that in the normal eggs (Figs. 5A, C, E and G). However, the diameters of spindles formed at metaphases I and II in the hybrid oocytes were larger than those in the normal *O. latipes* oocytes (Table 1, $p<0.01$, Student's *t*-test). The difference between the spindle diameter of the normal and hybrid oocytes is due to the difference in the number of chromosomes present in the oocytes, as demonstrated later.

To obtain further information on the behavior of chromosomes during meiosis, we observed maturing oocytes after staining with the DNA dye Hoechst 33258. In *O. latipes* oocytes, chromosomes were aligned at metaphase I and II plates (Figs. 4B and F) and equally separated into two groups at anaphase I and II (Figs. 4D and H); one remained in the oocyte cytoplasm and the other was included in the polar bodies and eliminated from the oocytes. Chromosomal behavior during meiosis in the hybrid oocytes (Figs. 5B, D, F and H) was similar to that in *O. latipes* oocytes (Figs. 4B, D, F and H), but the number of chromosomes in the hybrid oocytes was apparently larger than that in the normal oocytes (compare Fig. 4 and 5).

Changes in DNA content during meiosis in the hybrid medaka

Cytological observations of maturing oocytes of the hybrid medaka strongly suggest that meiosis takes place normally in the hybrid oocytes. However, it has been reported that unreduced egg formation in a triploid crucian carp is due to abnormal meiosis resulting in the absence of reduction in DNA content during meiosis (Yamashita *et al.*, 1993). To confirm actual reduction in DNA content during meiosis of the

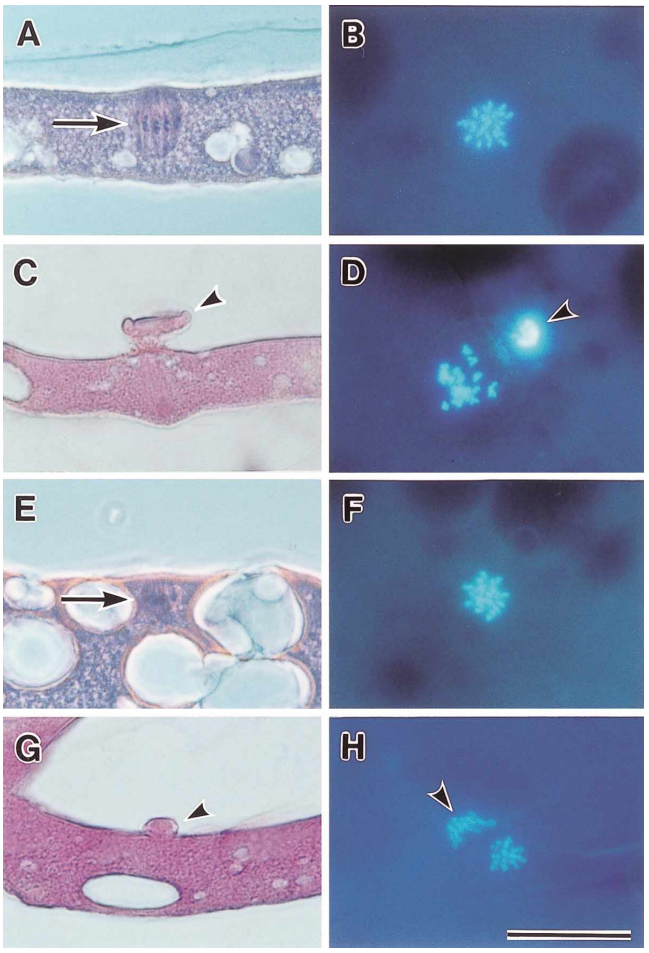

Fig. 4. HE-stained sections (A, C, E, G) and Hoechst 33258-stained specimens (B, D, F, H) showing spindles, polar bodies and chromosomes during meiosis in *O. latipes*. Metaphase I, A and B; anaphase I, C and D; metaphase II, E and F; anaphase II, G and H. Arrows show metaphase plates and arrowheads show polar bodies. Bar=25 μm.

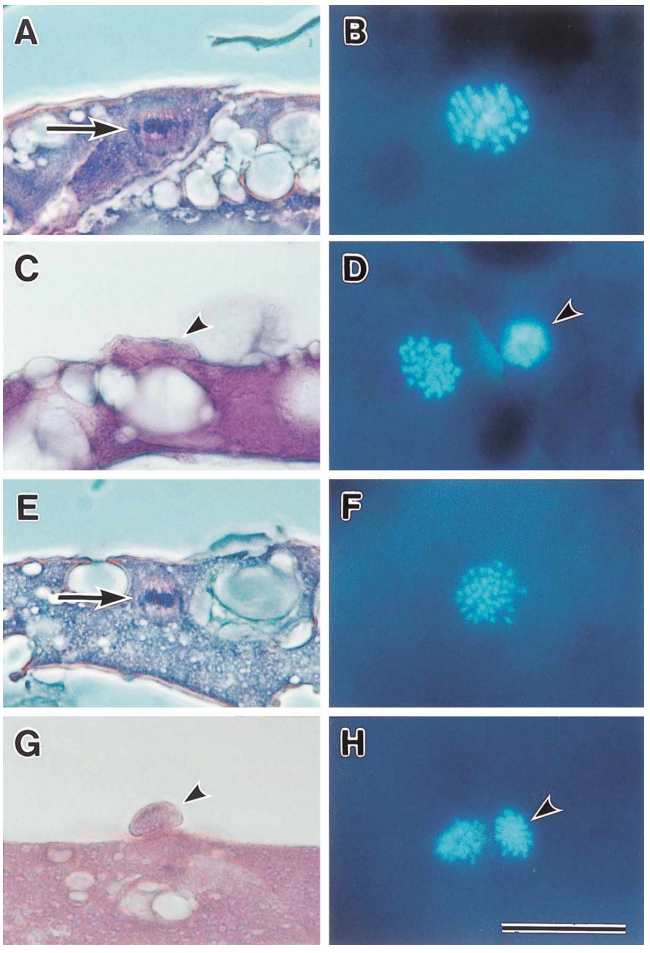

Fig. 5. HE-stained sections (A, C, E, G) and Hoechst 33258-stained specimens (B, D, F, H) showing spindles, polar bodies and chromosomes during meiosis in *O. latipes-curvinotus*. Metaphase I, A and B; anaphase I, C and D; metaphase II, E and F; anaphase II, G and H. Arrows show metaphase plates and arrowheads show polar bodies. Bar=25 μm.

Table 1. The diameter of spindles in the oocytes at meiotic metaphase I and II (μm)

	metaphase I	metaphase II
Oryzias latipes	8.88±0.08 (n=8)[a]	5.78±0.59 (n=3)[b]
O. latipes-curvinotus	11.31±0.47 (n=5)[c]	7.99±0.30 (n=4)[d]

The values are mean±standard error. Statistical significance: a–b, a–c, b–d, c–d ($p < 0.01$); a–d ($p > 0.05$).

hybrid oocytes, we therefore investigated changes in DNA content by measuring the intensity of Hoechst 33258 fluorescence.

The hybrid oocytes at anaphase II contained twice the amount of DNA as compared with that in *O. latipes* (Table 2, $p<0.01$), consistent with the previous notion that the hybrid produces diploid eggs (Hamaguchi and Sakaizumi, 1992; Sakaizumi *et al.*, 1992, 1993). DNA contents in the metaphase I and II oocytes in the hybrid were also significantly larger than those in *O. latipes* oocytes at the same phases (Table 2,

Table 2. Changes in DNA contents in the oocytes during meiosis (C)

	metaphase I	metaphase II	anaphase II
Oryzias latipes	4.59±0.21 (n=4)[a]	2.02±0.18 (n=6)[b]	1.00±0.15 (n=9)[c]
O. latipes-curvinotus	5.56±0.03 (n=2)[d]	3.23±0.31 (n=4)[e]	1.99±0.08 (n=2)[f]

The values (mean±standard error) are represented as a ratio to the intensity of *O. latipes* chromosomes at anaphase II. Statistical significance: a–b, a–c, b–c, b–e, c–f, d–f ($p < 0.01$); a–d, d–e ($p < 0.05$), e–f ($p > 0.05$).

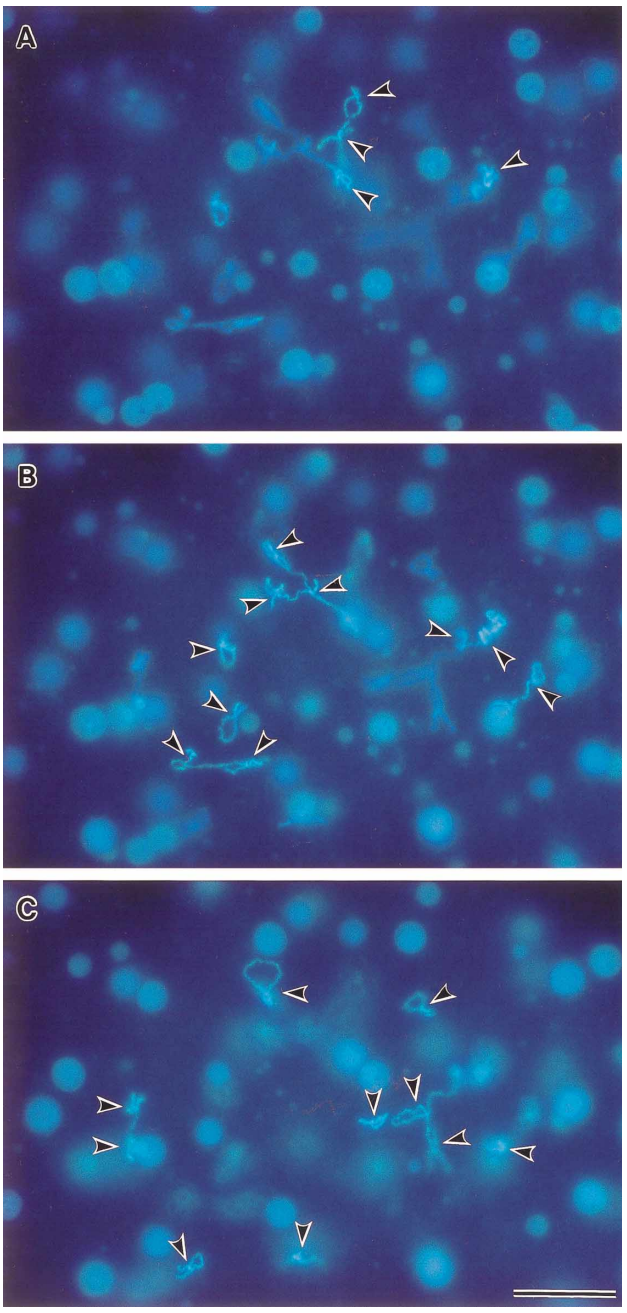

Fig. 6. A Hoechst 33258-stained germinal vesicle of *O. latipes* oocyte at the diplotene stage. Different focal planes (A–C) of the same specimen showing 24 bivalents (arrowheads). Bar = 25 μm.

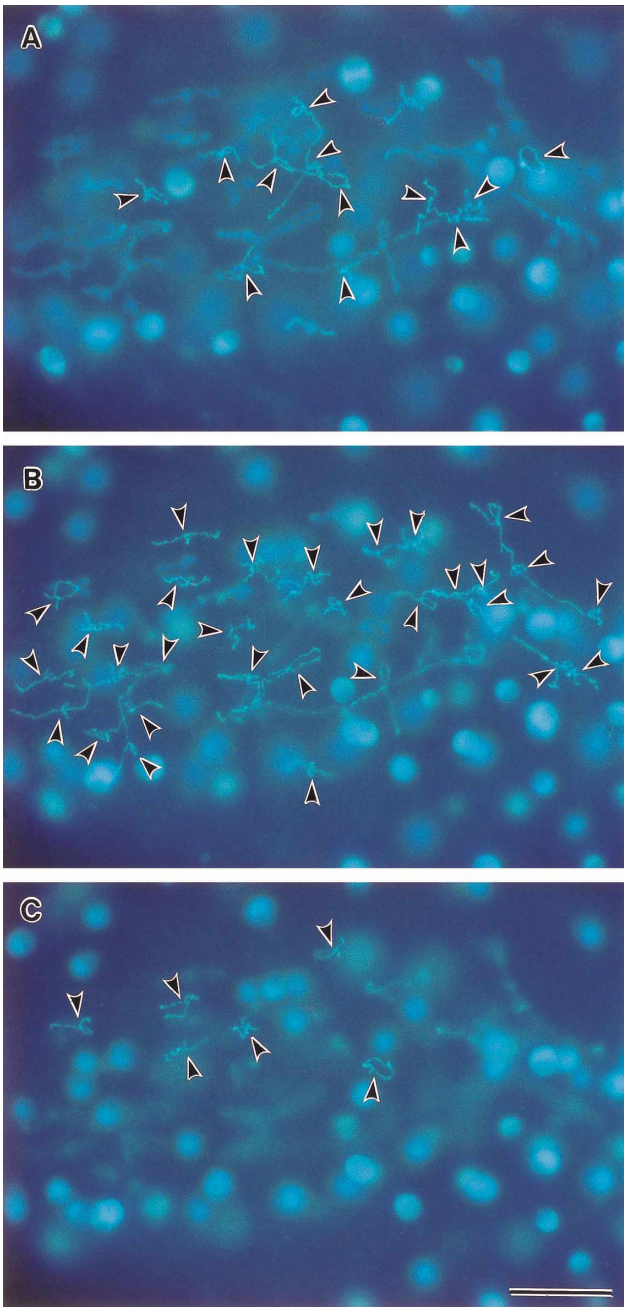

Fig. 7. A Hoechst 33258-stained germinal vesicle of *O. latipes-curvinotus* oocyte at the diplotene stage. Different focal planes (A–C) of the same specimen showing 48 bivalents (arrowheads). Bar = 25 μm

$p<0.05$ for metaphase I and $p<0.01$ for metaphase II). Furthermore, DNA content was clearly reduced during meiosis in the hybrid, as well as in *O. latipes*, although the measured values did not completely fit to the expected values, which are 4, 2 and 1 at metaphase I, metaphase II and anaphase II, respectively, for the normal oocytes and 8, 4 and 2 for the hybrid oocytes (Table 2).

Chromosome number in the hybrid oocytes

The DNA fluorometric study and the cytological observations indicated that there are already more chromosomes in hybrid oocytes at the beginning of meiosis and that meiosis itself proceeds normally with the reduction in DNA content by half after each of first and second meiotic divisions. To examine whether early-stage oocytes of the hybrid have an extra number of chromosomes, we counted the number of chromosomes in germinal vesicles of early diplotene oocytes. Each germinal vesicle of *O. latipes* contained 24 bivalent chromosomes having chiasmata, indicating that they are paired homologues (Fig. 6). The chromosomes in the hybrid oocytes were also bivalent with chiasmata, but their number was 48

(Fig. 7), precisely twice the number of chromosomes in the normal oocytes (Fig. 6).

DISCUSSION

Diploid eggs are produced by premeiotic endomitosis

Since the two sets of chromosomes in hybrids come from different species, pairing of homologous chromosomes is difficult. Indeed, our previous studies on spermatogenesis of the hybrid medaka between *O. latipes* and *O. curvinotus* indicated that most chromosomes do not pair at metaphase I (Shimizu *et al*., 1997). However, the present cytological observations together with DNA fluorometry have demonstrated that the hybrid oocytes are equipped with twice the number of chromosomes (48 vs. 24 in the normal oocytes) at the beginning of meiosis and that both first and second meiotic divisions proceed normally. We have also shown that all chromosomes in the germinal vesicles of the hybrid oocytes are bivalent with chiasmata, indicating that "homologous" chromosomes are present even in the hybrid. Taken together, the process of diploid egg production in the hybrid medaka is explainable as follows: First, oogonial chromosomes are doubled by endomitosis, the mitosis without cytokinesis, before entering meiosis, resulting in oogonia with pairs of identical chromosomes. Second, the tetraploid (4C) oogonia undergo premeiotic DNA replication and enter meiosis, producing oocytes with 8C DNA content, and, finally, normal meiotic division produces diploid (2C) eggs. A similar mechanism of unreduced egg formation has been found in *Xenopus laevis* and *X. gilli* hybrids (Kobel and DuPasquier, 1975) and in triploid fish (Cimino, 1972; Zhang *et al*., 1998) except for the triploid crucian carp *Carassius auratus langsdorfii* in which triploid eggs are produced by a defect in meiosis itself (Yamashita *et al*., 1993).

Type-B oogonia are connected by intercellular bridges (S. Hamaguchi, Niigata University, personal communication) and enter meiosis synchronously. Meiosis also proceeds synchronously. Thus, oocytes in normal medaka are observed as a cluster. In striking contrast to this, diplotene oocytes in the hybrid are found mostly as single cells of various sizes. These oocytes are probably derived from tetraploid oogonia produced by endomitosis. These findings suggest that after endomitosis the tetraploid oogonia do not divide mitotically any more. It is therefore likely that endomitosis occurs just before entry into meiosis.

Is endomitosis specific to hybrid females?

The female medaka constantly spawns a certain number of eggs every morning under appropriate conditions (Naruse *et al*., 1994). This indicates that the medaka ovary is equipped with a mechanism that organizes the entire process of oogenesis (proliferation of oogonia, entry into meiosis, vitellogenesis and maturation) by monitoring the number of germ cells at each process. The number of vitellogenic oocytes at the diplotene stage is considerably low in the hybrid ovary. If the above-proposed mechanism is in fact the mechanism that operates, it would force the hybrid ovary to produce oogonia and previtellogenic oocytes more actively in order to supply a sufficient amount of vitellogenic oocytes for coordinated oogenesis. Actually, the hybrid ovary contains unusually large numbers of previtellogenic oocytes, supporting the notion that early-stage germ cells are overproduced in the hybrid ovary. Endomitosis may occur only under such mis-regulated conditions. If so, endomitosis must be a phenomenon specific to the hybrid. In mammalian bone marrow, polyploid megakaryocytes (the precursors of platelets) are formed by endomitosis that is initiated by deficient activity of the M-phase-promoting kinase CDK1 due to the absence or very low levels of its regulatory subunit cyclin B1 (Vitrat *et al*., 1998). Whether similar mechanisms are responsible for endomitosis of the hybrid medaka oogonia remains to be investigated.

Another explanation for the occurrence of endomitosis in the hybrid is that endomitosis is not specific to the hybrid but occurs naturally in non-hybrid females at a very low frequency. Judging from the small number of the diplotene oocytes produced by endomitotic oogonia in the hybrid in which endomitosis is presumably enhanced by oogonial proliferation as discussed above, the frequency of endomitosis in the normal medaka, if it does occur, should be extremely low. In this case, it is unlikely that endomitosis itself causes any severe defects in viability or fecundity, thereby allowing the occurrence of endomitosis in the medaka from generation to generation. The spontaneous genomic duplication by inherent endomitosis may contribute to the evolution of medaka species to some extent. Further studies are required to verify whether endomitosis is the intrinsic characteristic of the normal medaka and, if so, we must clarify its biological significance.

Meiotic checkpoint differs in females and males

Most oocytes in the hybrid medaka cannot enter the pachytene stage of prophase I (present study, Hamaguchi and Sakaizumi, 1992). These oocytes fail to form paired chromosomes, as seen in spermatocytes (Shimizu *et al*., 1997). Synaptonemal complexes in these oocytes are also abnormal (Hamaguchi and Sakaizumi, 1992). On the other hand, endomitotically produced oocytes having paired chromosomes can enter the pachytene stage, as demonstrated in this study. Consequently, there must be a checkpoint monitoring the completion of chromosomal pairing at the zygotene stage. Several molecules that monitor recombination and synaptonemal complex formation have been discovered in some species. In the budding yeast *Saccharomyces cerevisiae*, mutations in molecules such as zip1, zip2, dmc1, sae3, and hop2 induce pachytene arrest (Bishop *et al*., 1992; Sym *et al*., 1993; McKee and Kleckner, 1997; Chua and Roeder, 1998; Tung and Roeder, 1998) and PCH2 is required for the meiotic checkpoint that prevents chromosome segregation when recombination and chromosome synapsis are defective (San-Segundo and Roeder, 1999). In addition, disruption of MLH1 or DMC1 in the mouse, which causes defective synapsis and/or recombination, results in the meiotic prophase I arrest (Edelmann *et al*., 1996; Pittman, *et al*., 1998; Yoshida *et al*., 1998). Since checkpoint mechanisms for proper meiotic chro-

mosome segregation at prophase I seem to be widely conserved, it is most likely that similar checkpoints are also active in medaka oocytes.

In contrast to oogenesis, we previously showed that checkpoints are not active at prophase I, but are active at metaphase I, in spermatogenesis (Shimizu *et al*., 1997). Meiotic metaphase arrest was probably caused through monitoring chromosome alignment at the spindle equator as in mitosis (Rieder and Alexander, 1989). Thus, it is likely that some meiosis-specific checkpoints are active in oogenesis but silent in spermatogenesis, and vice versa. In this context, it is notable that sex chromosome recombination is suppressed during medaka spermatogenesis regardless of the chromosomal condition; even in sex-reversed XX males, sex chromosome recombination is suppressed as it is in the XY male (Matsuda *et al*., 1999). Probably, gamete-type-specific activation of checkpoints and regulation of meiotic recombination are related to this phenomenon.

Ovarian architecture revealed by the hybrid ovary

The ovarian folliclular structures and their endocrine functions have been investigated in the medaka (Sakai *et al*., 1988; Nagahama, 1994; Iwamatsu and Nakashima, 1996). However, information about the somatic architecture of the medaka ovary is extremely limited (Yamamoto, 1955; Kanamori *et al*., 1985). The present study showed that a sac-like structure is one of the most prominent features of the hybrid ovary. Then, what component(s) of the ovary does the sac-like structure originate from? During normal development of the medaka ovary, type-B oogonia and early oocytes at the leptotene to pachytene stages are surrounded by somatic cells forming a cystic structure. Accordingly, the cyst contains synchronously developing germ cells but no somatic cells. The sac-like structure contains several somatic cells and locally grouped oocytes of various stages, suggesting that it includes several cysts. Therefore, the possibility that the sac-like structure represents expansion of the cyst by a large number of early-stage oocytes is excluded. Considering that all somatic elements develop and function normally in the hybrid, somatic cells forming the sac-like structure might not be an abnormal response. Rather, this structure must exist in the normal ovary with the same topology as that in the hybrid, but its outer shape would be deformed by numerous growing oocytes and cannot be observed.

In conclusion, we found that diploid eggs laid by the hybrid female medaka are formed through normal meiosis from endomitotically produced tetraploid oogonia. We also revealed a difference in the checkpoints of meiosis between the male and female hybrids and the basic ovarian architecture that is difficult to define in the normal ovary. Given that the hybrid male is also a useful tool (Shimizu *et al*., 1997), our hybrid medaka furnishes a powerful experimental system for investigating the regulatory mechanisms of gametogenesis and the fundamental structure of gonads.

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