THE TIMING OF MEIOSIS AND DNA SYNTHESIS DURING EARLY OOGENESIS IN THE TOAD, *XENOPUS LAEVIS*

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ABSTRACT

Recently metamorphosed female *Xenopus laevis* toads were injected with tritiated thymidine. Animals were kept at 20°C and were sacrificed 1–23 days after isotope injection. Radioautographs of squash preparations of the ovaries were made. The progress of labeled germ cell nuclei was followed to obtain information on the time course of early meiosis and extrachromosomal DNA synthesis. Premeiotic S was estimated to take not more than 7 days. Leptotene takes 4 days, zygotene takes 5 days, and pachytene was estimated to be completed in about 18 days. The major period of amplification of the extrachromosomal DNA occurs in pachytene and takes about 13 days. A low level of synthesis was observed before and after this period, in zygotene and late pachytene–early diplotene, extending the total time for extrachromosomal DNA synthesis during meiosis to about 18 days. These data allowed the calculation to be made that one round of replication of the amplified DNA takes between 1.2 and 3.0 days. It was also found that in both oogonial and premeiotic interphases, the nucleolus-associated DNA shows asynchronous (probably late) labeling with respect to the chromosomes.

INTRODUCTION

During oogenesis in the toad, *Xenopus laevis*, the nuclei of germ cells show the normal mitotic and meiotic sequences of chromosome replication, division, and pairing, found in the majority of eukaryotic organisms and in addition, a specific amplification of the genes coding for ribosomal RNA (rRNA) (2, 6). Gene amplification in the female germ line has been well characterized in some lower vertebrates and certain insects (7). In *Xenopus*, the major differential synthesis of ribosomal cistrons occurs during the pachytene stage of meiosis, and results in a threefold increase in the DNA content of the oocyte nucleus (11). At this time the chromosomes are typical paired homologues arranged in a bouquet configuration. The amplified DNA is present in the nucleus as a cap of dense material, first described in Amphibia by King (10) in 1908 as "chromatin not used for the chromosomes." Later, Painter and Taylor (13) showed that this material is Feulgen-positive, and it has since been shown that in *Xenopus* the cap incorporates tritiated thymidine during formation (15). Cytological hybridization has recently been used to demonstrate that rRNA is specifically bound in the cap region of pachytene oocytes, confirming the nature and localization of the amplified DNA (8). In a later stage of meiosis, diplotene, up to 1500 nucleoli are formed in

association with the ribosomal DNA (rDNA) (5, 15) but free of the chromosomes which are in the "lampbrush" stage. Thus the amplified DNA is extrachromosomal and functions independently in rRNA synthesis.

The present study was undertaken to provide information on the time course of early meiosis and the length of the period during which the extrachromosomal DNA is amplified in young *X. laevis* toads. This was done by following the progress of radioactively labeled nuclei through oogenesis. A somewhat similar study was recently carried out by Bird and Birnstiel (1).

MATERIALS AND METHODS

Tadpoles of *X. laevis* (Daudin), the South African clawed toad, were raised from eggs at 20°C in the laboratory. As only ovaries were required for this study, animals were treated from stage 40 (12) with estradiol (50 μg/liter, Calbiochem, Los Angeles, Calif.) which produces ovaries in genetic males (9).

2–10 μCi of thymidine-methyl-^{3}H (SA 11.3 Ci/mmole, Schwarz Bio Research Inc., Orangeburg, N. Y.) were injected into the body cavity of young toads, 2–3 wk after metamorphosis. Animals were kept at 20°C, and were sacrificed 1–23 days after isotope injection. One or two animals were used each day. The ovaries, which contain mostly early meiotic and premeiotic stages in young animals, were dissected out and fixed in ethanol–acetic acid (3:1), followed by 45% acetic acid. They were squashed between a cover slip and a microscope slide and frozen rapidly on a flat block of dry ice. The cover slip was removed with a razor blade. The slides were placed in 70% ethanol for a few minutes, then air dried.

Slides were coated with NTB-2 emulsion (Kodak) diluted 1:1 with distilled water and were exposed in light-proof boxes for 7–33 days. The radioautographs were developed with D19 for 2 min, fixed, stained with Giemsa, rinsed, dried, and mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.). Photographs were taken with a camera on a Zeiss microscope (Carl Zeiss, Inc., New York), using Adox KB 14 or Kodak High Contrast Copy film. Prints were made with Agfa-Gevaert Brovira paper.

RESULTS

The time taken for cells to pass through different stages of a cell cycle can be determined by labeling the cells at one time and observing their progress at successively later times. Labeling is carried out with tritiated thymidine which is metabolically stable after incorporation into DNA. The first labeled cells to reach a particular stage are those that incorporated thymidine at the end of the preceding DNA replication period. In the case of meiosis, the time taken by cells to proceed from the end of premeiotic S phase to the beginning of a later stage can be estimated by observing the time that elapses between administration of isotope and the first occurrence of labeled cells in the stage under consideration.

TABLE I

Distribution of Label over Nuclei Progressing through Oogenesis in the Toad, Xenopus laevis, Tabulated as Time (in days) after Injection of Tritiated Thymidine

Stage	Chromosomal label	Nucleolar label	Cap label
Oogonia	1–6	1–5	
Premeiotic interphase-leptotene	1–10	1–5	
Zygotene	5*	1, 2 12, 14	
Pachytene 15 μ	10*		1–5 (6–9)
Pachytene 20 μ	14*		1–5 (6–9)
Pachytene 20–30 μ	19*		1–12
Pachytene 30–40 μ	23		1*
Late pachytene 40–50 μ			5*
Diplotene 50–60 μ			10*
Diplotene 60 μ+			14*

Figures in parentheses refer to days on which only a low level of label is observed. Measurements in microns refer to average nuclear diameters in moderately squashed preparations and are used to indicate the general progress of oocytes through pachytene and diplotene. In pachytene, the proportion of the nucleus occupied by the cap was used as an additional criterion.

* Indicates that label was observed from the day given until the end of the experiment (23 days).

In this study, the progress of chromosomal label was used to determine the length of leptotene, zygotene, and early and mid-pachytene. By the end of the experiment (23 days after injection of isotope) the most advanced labeled cells were in mid-pachytene. Since the uptake of thymidine into extrachromosomal DNA has been shown to be distinct in timing and localization in *Xenopus* oocytes, the progress of this label was followed

in an analogous fashion to provide additional information about late pachytene and early diplotene. The results are summarized in Table I.

Early Oogonia

In squash preparations, early oogonia have large, irregularly shaped (frequently doughnut-shaped) nuclei. They usually contain more than the expected diploid number of two nucleoli. Most often four or five nucleoli are present, but as many as nine nucleoli per nucleus have been seen in these preparations. Cytological hybridization experiments have previously demonstrated that each oogonial nucleolus binds rRNA, indicating that some amplification of the ribosomal cistrons is present even at this early stage in oogenesis (14).

1 day after isotope injection, many oogonial nuclei remain unlabeled. However, some nuclei show heavy chromosomal label (Fig. 1) and must have been in an oogonial S phase when tritiated thymidine was available. It is not possible to determine the length of oogonial stages directly from the data on chromosomal labeling in this experiment.

Nuclei in which the nucleoli are labeled are also found 1 day after isotope incorporation. Some of these nuclei also show label over the chromosomes; in others only the nucleoli are labeled. In an oogonial nucleus with several nucleoli, it is evident that all the nucleoli are labeled (Fig. 2). It is also found that after 1 day oogonial mitotic prophases are present which contain exclusively nucleolar label (Fig. 3). Unless oogonial S phases are shorter than 1 day, the first nuclei to reach mitosis are those which incorporated label into the nucleoli late in the previous S phase. This suggests that the nucleolus-associated DNA, some of which may be present in the nucleus as amplified rDNA cistrons, continues to incorporate tritiated thymidine after the bulk of the chromosomal DNA has completed its replication.

Premeiotic Interphase–Leptotene

After the last oogonial mitosis, the germ cells enter the premeiotic (or oocyte) interphase. The cells undergo DNA replication in the premeiotic S phase, then enter leptotene. In squash preparations, cells in leptotene and premeiotic interphase have round nuclei which contain one or two (rarely three or more) centrally located nucleoli. Although these nuclei usually contain the number of nucleoli expected in a diploid cell, the presence of some amplification of the ribosomal cistrons has previously been indicated by cytological hybridization studies (7). It is evident in the electron microscope that cells in this stage are found in groups of about 16 cells, presumably derived from a series of oogonial mitoses. Each group, or cell nest, develops synchronously until late pachytene when the cells become separated (4).

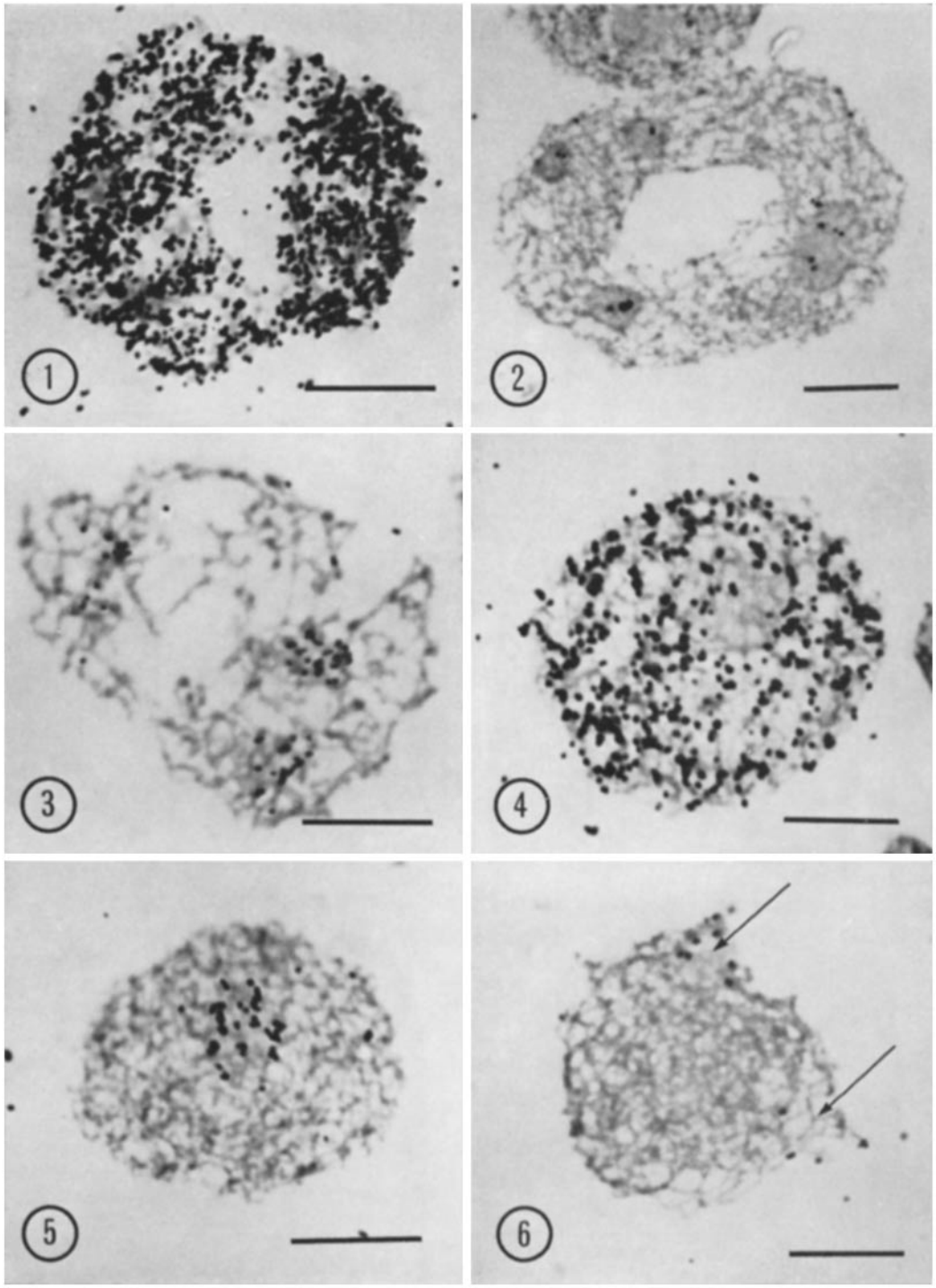
1 day after injection, some nuclei show heavy label over the chromosomes (Fig. 4). These nuclei were evidently in premeiotic S when thymidine was available. The nucleoli are usually relatively unlabeled, but in some nuclei both the chromosomes and nucleoli are heavily labeled. Some nuclei are observed with only the nucleoli labeled (Fig. 5), suggesting that asynchronous labeling of the nucleolus-associated DNA occurs also during premeiotic S (7). Many unlabeled nuclei are also present, representing cells that were not replicating DNA when isotope was available. Some of these nuclei have relatively condensed chromosomes and could be in leptotene of meiosis.

As cells progress into meiosis with time there is a decrease in the number of labeled nuclei in leptotene and premeiotic interphase stages (although there is some addition from the labeled oogonial population). Labeled nuclei begin to appear in progressively later stages with increasing time after isotope administration.

Zygotene

Zygotene nuclei are characterized by condensed chromosomes oriented in a bouquet configuration. Synapsis starts at the chromosome ends which are directed towards one side of the nucleus. One or two round nucleoli are located eccentrically on the side of the nucleus away from the chromosome ends.

Up to 4 days after injection, zygotene nuclei remain mostly unlabeled. In some nuclei a few grains (above the background level) are seen over the chromosomes. This incorporation of thymidine may reflect a low level of DNA synthesis which has been shown to be necessary for synapsis in some plants (16). Wimber and Prensky (17) concluded from labeling experiments that incorporation in zygotene had occurred during male meiosis in the amphibian, *Triturus viridescens*.



FIGURES 1–6 1 day after injection of 5 μCi thymidine-^{3}H; radioautographs exposed 7 days. Fig. 1: Oogonial nucleus, chromosomal label. × 1900. Fig. 2: Oogonial nucleus, nucleolar label. × 1400. Fig. 3: Oogonial nucleus in mitotic prophase, nucleolar label. × 1900. Fig. 4: Premeiotic interphase nucleus, chromosomal label. × 1700. Fig. 5: Premeiotic interphase nucleus, nucleolar label. × 1900. Fig. 6: Zygotene nucleus, nucleoli (arrows) labeled. × 1700. Scale = 10 μ.

However, Callan and Taylor (3) could not find direct evidence of thymidine incorporation at this stage in a study of a closely related species, *T. vulgaris*.

5 days after thymidine incorporation, some zygotene nuclei first show heavy chromosomal label, which must have been taken up during the premeiotic S phase (Fig. 7). It is concluded that these nuclei took 4 days to pass through leptotene.

A low level of nucleolar label is also seen in some zygotene nuclei during the first few days after isotope injection (Fig. 6). This is evidence for the replication of rDNA in zygotene, and suggests that amplification occurs at a low level before the start of pachytene. After the first few days of the experiment it is possible that some nuclei with nucleolar label could have progressed to zygotene from premeiotic interphase, but it is felt that the nucleolar label seen so soon after injection does represent incorporation in zygotene. Since not all zygotene nuclei show nucleolar label, amplification of the ribosomal cistrons does not occur at a detectable rate for at least part of zygotene.

The proportion of nuclei with nucleolar label decreases rapidly after a few days following injection, and by 1 wk no nucleolar label is seen in zygotene nuclei. On days 12 and 14, however, nuclei in zygotene with nucleolar label are again seen in the preparations (Fig. 8). This label must have been incorporated asynchronously into the nucleolus-associated DNA at some time before the last premeiotic chromosome replication period. It cannot be determined conclusively whether these nucleoli labeled early in premeiotic S or late in the last oogonial S phase. In either case, the premeiotic S phase can be no longer than 7 days (i.e., 12 days minus the 5 days from the end of the premeiotic S phase to the beginning of zygotene). If the nucleoli are late labeling (which seems probable), this figure must include the time for the oogonial G_2, mitosis and the oocyte G_1, as well as premeiotic S.

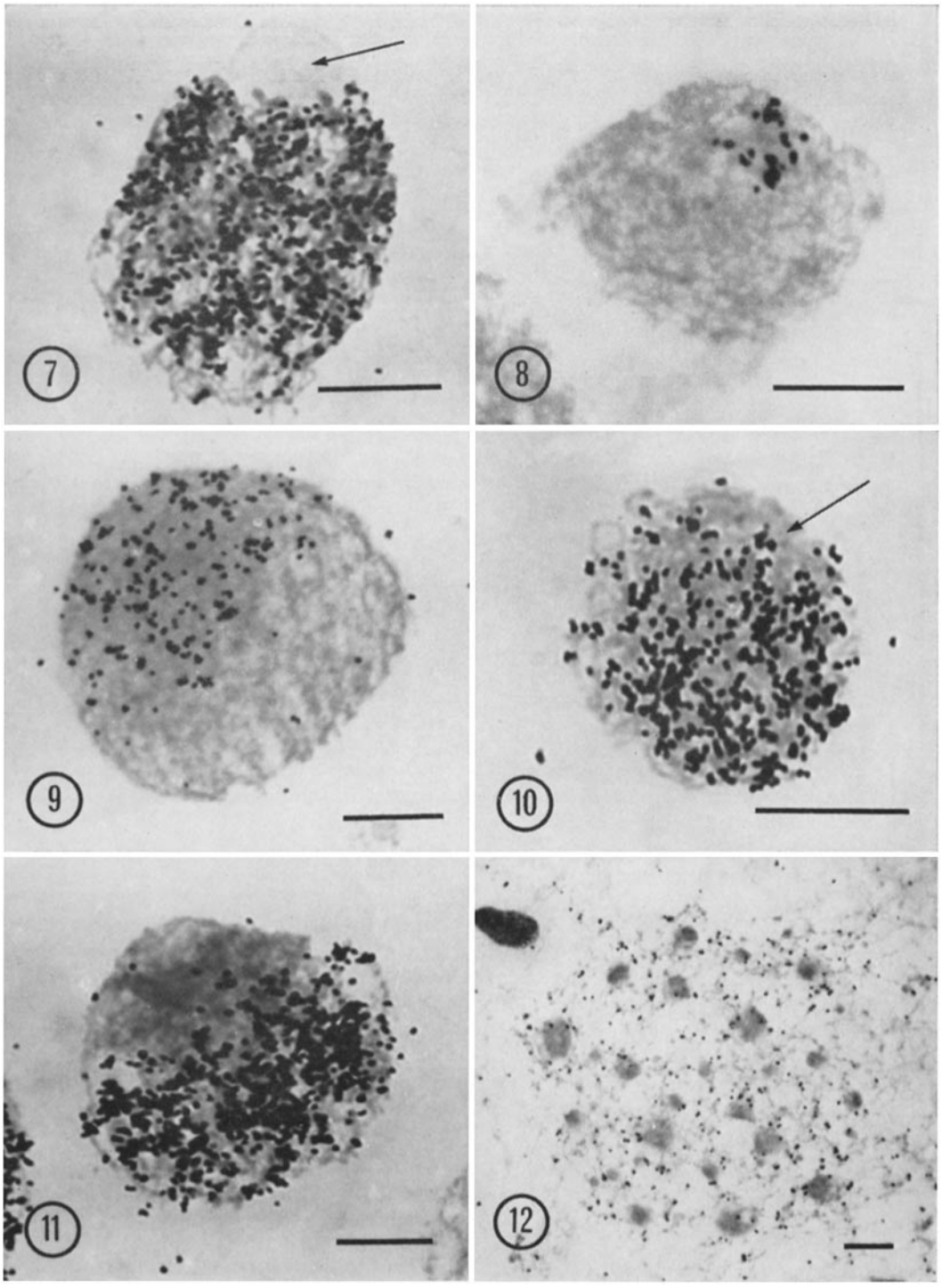
Pachytene

The major period of amplification of extrachromosomal DNA in *X. laevis* oocytes occurs in pachytene.[1] In the smallest pachytene nuclei, 15 μ in diameter, the cap is a thin crescent of material lying around an irregularly shaped nucleolus (4). The cap is heavily labeled up to 5 days after injection, but by day 6 the number of labeled early pachytene nuclei decreases. By day 10, nuclei in this stage no longer show label over the cap, but some now show heavy chromosomal label (Fig. 10), indicating that 9 days have elapsed between the end of premeiotic S and the start of pachytene. Since leptotene has been shown to take 4 days, zygotene must occupy 5 days.

As pachytene proceeds, the nuclei increase in size. The cap also occupies a relatively greater proportion of the nucleus as amplification of the rDNA cistrons continues. Pachytene nuclei, up to about 40 μ in diameter, show heavy label over the cap after isotope administration (Fig. 9), but after some days cap label is no longer present. This occurs first in the earliest pachytene nuclei and is due to unlabeled nuclei (in zygotene or leptotene when isotope was available) entering the stage. Older pachytene stages show cap label for a longer period of time, while nuclei labeled earlier in pachytene still progress through, but eventually unlabeled nuclei enter the population. Subsequently, chromosomal label (incorporated in premeiotic S) appears in pachytene stage nuclei. (Chromosomal and cap label are never found together in the same pachytene nucleus.) As before, the first appearance of chromosomal label reveals the time taken from premeiotic S to the stage. For pachytene nuclei 20 μ in diameter, chromosomal label is seen on day 14; for 20–30 μ diameter nuclei, on day 19 (Fig. 11); for 30–40 μ diameter nuclei, on day 23. Thus pachytene, until nuclei reach up to 40 μ in diameter, takes 13 (23 minus 10) days. By the end of the experiment, nuclei of 40 μ or greater diameter had yet to show chromosomal label. Labeling of the cap was therefore examined to provide further information about late pachytene and early diplotene.

Nuclei of 40–50 μ diameter are in late pachytene or early diplotene, but exact staging is difficult in squash preparations as both the chromosomes and the cap are becoming increasingly dispersed. One day after isotope injection these nuclei show either a low level of incorporation or no label in the cap region, which suggests that by this stage replication of the extrachromosomal DNA is almost completed. Heavy cap label is seen in these nuclei by day 5, and two conclusions can be drawn from this observation. First, it adds

[1] In squash preparations, pachytene nuclei are characterized by completely paired chromosomes in a bouquet configuration and by a cap of extrachromosomal DNA.



FIGURES 7 and 8 2 μCi thymidine-^{3}H; exposed 33 days. Fig. 7: 5 days. Zygotene nucleus, chromosomal label, nucleolus (arrow) unlabeled. × 1800. Fig. 8: 12 days. Zygotene nucleus, nucleolar label. × 1900.

FIGURE 9 1 day after injection of 5 μCi thymidine-^{3}H; exposed 7 days. Pachytene nucleus, cap label. × 1500.

FIGURES 10–12 2 μCi thymidine-^{3}H; exposed 33 days. Fig. 10: 10 days. Early pachytene nucleus, chromosomal label, cap (arrow) unlabeled. × 2250. Fig. 11: 19 days. Pachytene nucleus, chromosomal label. × 1360. Fig. 12: 19 days. Cap region of diplotene nucleus, labeled extrachromosomal DNA around nucleoli. × 700. Scale = 10 μ.

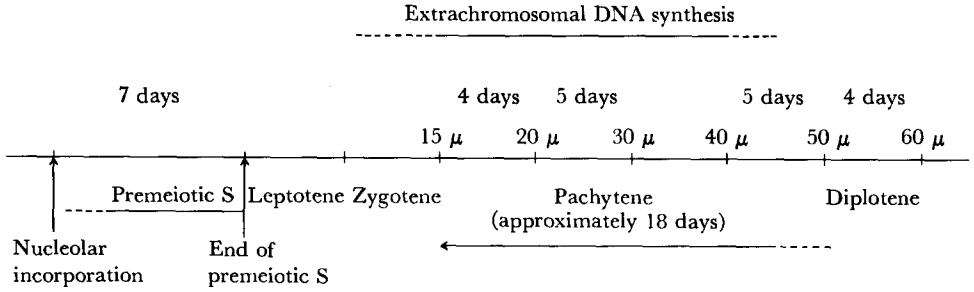


FIGURE 13 The time course of meiosis in *X. laevis* oogenesis at 20°C.

about 5 days to the time that cells spend in pachytene, giving a total of 18 days. Second, the major period of extrachromosomal DNA synthesis is about 5 days shorter than pachytene, that is about 13 days.

Diplotene

Diplotene nuclei are characterized by the presence of lampbrush chromosomes. The extrachromosomal DNA is initially a cap of material, less compact than in pachytene, which becomes more uniformly distributed through the nucleus as the stage progresses. Multiple nucleoli are formed in association with the cap. Diplotene nuclei, 50–60 μ in diameter, are unlabeled until day 10 when extrachromosomal DNA (i.e., cap) label is found associated with the nucleoli (Fig. 12). This label was incorporated in late pachytene. It therefore takes 5 (10 minus 5) days for the nuclei to increase from 40–50 μ to 50–60 μ diameter. In a further 4 days nuclei larger than 60 μ are labeled over the extrachromosomal DNA. Studies on sectioned material show that a large increase in cytoplasmic volume accompanies the growth in nuclear volume. The diplotene cell continues to grow for several months, eventually reaching 1 mm or more in diameter.

DISCUSSION

The conclusions drawn from this study are summarized in Fig. 13. At 20°C, in ovaries from recently metamorphosed *X. laevis* toads, premeiotic S takes a maximum of 7 days, leptotene takes 4 days, zygotene 5 days and pachytene about 18 days. The major synthesis of extrachromosomal DNA occurs in early and mid-pachytene and takes about 13 days. A low level of synthesis also takes place before and after this period, in zygotene and late pachytene–early diplotene, extending the total time of synthesis by a few days to about 18 days.

The timing of male meiosis in *X. laevis* has not been reported in the literature. In the urodele *T. vulgaris*, Callan and Taylor (3) showed that during spermatogenesis premeiotic S took 9–10 days, leptotene lasted 5 days, zygotene 8 days, pachytene 4–5 days, and diplotene 1–2 days. Although these data are from a different species, they do afford some basis for a comparison of male and female meiosis in Amphibia. It is evident that the ratio of pachytene (and diplotene) to the combined period of leptotene and zygotene is much greater in oogenesis than in spermatogenesis. The extended time that the oocyte spends in diplotene is undoubtedly due to the need to accommodate the synthesis of storage materials for the egg. The longer pachytene period allows some increase in oocyte size, but may also be correlated with the synthesis of the extrachromosomal DNA at this time.

This study of the time required for amplification of the rDNA allows estimates to be made of the time taken for an average round of replication of the extrachromosomal DNA at 20°C. The calculation is based on the assumption that amplification is carried out by a cascade mechanism, with successive rounds of synthesis in each of which every molecule of rDNA present in the nucleus acts as a template once. It has been estimated (15) that about 11 rounds of synthesis would be necessary to produce the final number of copies from the original nucleolus organizers in *X. laevis*. Using this figure, and assuming that all synthesis occurs during the major period of rDNA amplification, we arrive at 1.2 days (13 days/11 rounds) for an average round of replication. This calculation can be modified to yield a larger, and possibly more realistic, estimate by taking an additional factor

into account. Evidence from cytological hybridization suggests that about 20–40 copies of the genes for rRNA may be present already by premeiotic interphase (8), so that the first five rounds of synthesis ($2^5 = 32$) may be completed before the start of pachytene. A high estimate can then be made by assuming that only six rounds of synthesis occur during the major period of DNA amplification, and by using the longest estimate of 18 days for this period. This gives 3.0 days (18 days/6 rounds) for a round of synthesis. It is concluded that an average round of replication during the major period of rDNA amplification takes between 1.2 and 3.0 days.

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