OBSERVATIONS ON EARLY GERM CELL DEVELOPMENT AND PREMEIOTIC RIBOSOMAL DNA AMPLIFICATION IN *XENOPUS LAEVIS*

MARVIN R. KALT and JOSEPH G. GALL

From the Department of Anatomy, University of Connecticut Health Center, Farmington, Connecticut 06032, and the Department of Biology, Yale University, New Haven, Connecticut 06520

ABSTRACT

The origin of premeiotic ribosomal DNA (rDNA) amplification in germ-line cells of *Xenopus laevis* has been examined using *in situ* RNA-DNA hybridization on cytological preparations, tritiated thymidine autoradiography, and isopycnic density gradient centrifugation. Primordial germ cells (PGC), from the time they first become localized in the genital ridge at day no. 4 of development, until approximately day no. 22, remain in an extended interphase condition. During this time PGC do not incorporate tritiated thymidine, have near diploid levels of rDNA as demonstrated by cytological RNA-DNA hybridization, and possess only one or two nucleoli. Starting on day no. 22–24, mitosis, sexual differentiation, and rDNA gene amplification all begin in the germ cells. Multiple nucleoli also make their appearance at this stage. Ribosomal DNA amplification continues in gonial cells as long as they remain mitotically active. Amplified copies of rDNA are lost from germ cells at the onset of meiotic prophase. This loss is probably permanent in the male germ line, but variable and temporary in the female germ line. Early gonial cells in the ovary have been deduced to have an average cycle time for each mitotic division of between 3.8 and 4.3 days at a temperature of 21°C. Some oogonia appear to divide only four times before entering meiotic prophase, while the average during the initial wave of germ cell division is nine. Finally, a satellite DNA has been isolated from adult testes which has a density in neutral cesium chloride corresponding to the density of amplified oocyte rDNA. This satellite is not present in DNA isolated from somatic tissues of *Xenopus*.

INTRODUCTION

Over a period of several weeks during early meiotic prophase in oocyte nuclei of the amphibian *Xenopus laevis*, a 2,500-fold increase occurs in the number of genes coding for ribosomal RNA (4–6, 12, 13, 19). These genes function as templates throughout oogenesis to produce the enormous amount of ribosomal RNA[1] present in the mature amphibian egg (9). This meiotic period of ribo-

[1] *Abbreviations used in this paper:* DNA, deoxyribonucleic acid; PGC, primordial germ cell; rDNA, DNA containing the sequences coding for 7, 18, and 28S

somal DNA gene amplification, however, is not the first increase in the number of ribosomal cistrons to occur in the germ line of *Xenopus*. Gall and Pardue (14), utilizing the technique of *in situ* RNA-DNA hybridization on cytological preparations, discovered that a low level of rDNA gene amplification (ca. 5–20 nucleolus organizer equivalents) existed in mitotically active oogonia of newly metamorphosed animals. In addition, it was also noted that such gonial cells possessed as many as nine nucleoli, well in excess of the one or two nucleoli present in the somatic cells of the organism. More surprising was the finding that rDNA amplification and multiple nucleoli were also present in the spermatogonia of the male germ line of *Xenopus* (21). Subsequent investigation by Pardue and Gall (22) revealed that this preliminary amplification is lost at the onset of meiotic prophase in the male germ line.

The present investigation is directed toward the study of this gonial or premeiotic phase of rDNA amplification. Specifically, two aspects of this problem have been examined. First, when in the cycle of germ cell development does ribosomal DNA amplification begin? And second, is the gonial amplification process in the male germ line related to the meiotic amplification process in the oocyte? These questions have been examined utilizing the techniques of *in situ* RNA-DNA hybridization on cytological preparations, tritiated thymidine autoradiography, and isopycnic density gradient centrifugation. The results of this study suggest that the early primordial germ cells of *Xenopus* contain neither amplified copies of rDNA nor multiple nucleoli. The start of the amplification process appears to be concomitant with the onset of sexual differentiation and mitosis in the germ cells of the tadpole. The cell cycle of early female gonial cells has been estimated, and is longer than the time required for one round of replication of amplified rDNA (2, 27). *In situ* RNA-DNA hybridization on cytological preparations indicates that germ cells in both sexes probably lose amplified rDNA at the onset of meiotic prophase, although this loss does not appear to be total in the female. Finally, analytical ultracentrifugation experiments suggest that the testis contains a DNA satellite which has the same density in neutral cesium chloride as the amplified rDNA found in oocytes.

ribosomal RNA plus a nontranscribed "spacer" region; RNA, ribonucleic acid; SSC, 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7; [^{3}H]TdR, tritiated thymidine.

MATERIALS AND METHODS

Animals

Tadpoles of *Xenopus laevis* reared in captivity are subject to wide fluctuations in rate of development due to factors such as crowding, temperature, diet, and genetic background. Depending on these variables, development from zygote to metamorphosis may take as little as 6 wk or as long as 6 mo. To minimize variability, the following protocol was established. All animals were raised at 21 ± 1°C at an initial concentration of 10 tadpoles/liter, and were fed daily on nettle powder mixed with bonemeal. The first tadpoles to reach Nieuwkoop-Faber stage 50 (20) were sorted out, matched for size and external characteristics, and were placed at a concentration of two tadpoles/liter. In some groups, 17 β estradiol (Sigma Chemical Co., St. Louis, Mo.) was added to the water at a concentration of 50 μg/ml to induce feminization (16). All animals were fed daily and examined for external stage characteristics. Only the most advanced tadpoles were retained for study. This protocol allowed synchronization of development to within 1–2 days for any given stage. For experiments using adult gonads, animals at least several years old were selected from wild stocks.

Autoradiographic Procedures

INCORPORATION STUDIES: 2–5 μCi tritiated thymidine (NET-027Z, specific activity 40–50 Ci/mM, New England Nuclear, Boston, Mass.) were injected intraperitoneally into tadpoles and young frogs to assay whether cells at a given stage were synthesizing DNA. Animals were sacrificed 24 h later and gonads were processed for examination as described below.

TOTAL CELL CYCLE DETERMINATIONS: The number of synthetically active gonial cells was determined by "continuous" labeling with [^{3}H]TdR. Intervals between injections were determined empirically until 100% cell labeling or a 48-h plateau in percent cells labeled had been reached. As a result of these tests, animals were given 1–2-μCi injections twice daily and were sacrificed 2–8 days after the start of treatment. Classification of cell stages was made according to the developmental stage at time of sacrifice.

IN SITU RNA-DNA HYBRIDIZATION ON CYTOLOGICAL PREPARATIONS: Squashes of gonads from stage 43 to adult were hybridized with tritiated RNA which had been transcribed from purified *Xenopus* amplified rDNA in vitro using *Escherichia coli* RNA polymerase (15). The complementary RNA had a specific activity of 10^8 dpm/μg, and was used at a concentration of 6×10^6 cpm/ml. 20–30 μl of complement were used per slide. Hybridization and reactions were carried

out as described by Gall and Pardue (15), with the exception that acid extraction of the tissue was omitted. Slides were exposed 4–8 wk before development.

In all experiments, gonads were dissected out of animals and were processed for light microscope autoradiography either by squashing (15) or by sectioning. For thymidine incorporation studies, tissues were treated with cold 5% trichloroacetic acid before being processed for autoradiography according to standard procedures (23). RNase and DNase digestion was performed on control slides to ensure that no transfer of radioactive label had occurred. Slides were stained with either Giemsa or 1% toluidine blue in 1% borax.

DNA Isolation and Characterization

DNA isolated from *Xenopus* testes and liver was examined for the presence of heavy (high guanine + cytosine) satellites. Testes were taken from adult males which had received 250 U of human chorionic gonadotropin (Sigma Chemical Co.) and had been mated 3 days before sacrifice. Liver DNA was extracted from the same animals. DNA was obtained from each tissue by Sarkosyl-EDTA-pronase treatment followed by deproteinization with phenol and digestion with RNase and α-amylase according to the procedure of Barsacchi and Gall (1). After isolation, the DNA was dissolved in a small volume of 0.1 × SSC and was mixed with saturated cesium chloride to give a solution with a final density of 1.70 g/cm^3. A total of 500 μg of DNA was loaded in eight 4.5-ml gradients. These were then centrifuged to equilibrium at 20°C in a Spinco L2 50 preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using an A1 50 fixed-angle rotor. Gradients were collected in 10-drop fractions and DNA was assayed in a spectrophotometer at 260 nm. Fractions to the heavy side of the main peak as determined by optical density were pooled, reprecipitated with 70% ethanol, then redissolved in an 0.4-ml cesium chloride gradient with a final density of 1.70 g/cm^3. In some cases, 0.1–0.5 μg mouse satellite DNA, ρ = 1.690 g/cm^3 (11) was added to the *Xenopus* DNA as a density marker. The gradient was then centrifuged to equilibrium in a Spinco model E analytical ultracentrifuge in a 2° single sector cell. After equilibrium had been reached (44,770 rpm for 20 h at 20°C in an ANG rotor), the samples were photographed with ultraviolet optics, and the resulting negative was traced with a Joyce-Loebl recording microdensitometer (Joyce, Loebl & Co., Inc., Burlington, Mass.). Densities of satellites were determined with respect either to the mouse marker or to *Xenopus* main peak DNA, ρ = 1.699 g/cm^3 (25).

RESULTS

Sequence of Germ Cell Development

The primordial germ cells of *Xenopus* first become localized in the developing genital ridge of the tadpole during the 4th day of development at stage 43 (20). By day no. 8 of development (stage 48), the original single ridge forms the sexually undifferentiated, paired gonadal rudiments (Fig. 1). Depending on clutch and individual, each rudiment contains from 9 to 16 PGC. PGC number remains constant until stage 52, which occurs between day no. 22 and day no. 25 of development under the growth conditions used in this study. This indicates that the germ cells do not divide during this period, in general agreement with previous studies (8, 20, 24). This dormancy is further documented by the finding that PGC fail to label with tritiated thymidine during most of this period (Table I).

Toward the latter portion of stage 51, labeling of some germ cell nuclei with [^{3}H]TdR is first observed, and the first increase in germ cell number is recorded (Table I). The germ cells, now termed "gonia," do not label simultaneously, sections revealing that the gonia in the anterior portion of the gonad tend to label earlier than those in the posterior region. Table I indicates that at day no. 22 a maximum of 30% of gonia have incorporated thymidine into DNA after 7 days of continuous labeling. The sexual differentiation of the gonads also begins at stage 52 (18, 20).

By stage 53, which extends from day no. 25 to day no. 29 of development, increased numbers of nucleoli are observed in some gonial cells (18, 24). Electron microscope examination reveals that in addition to the typical fibrogranular nucleoli present in PGC, there are several smaller, largely fibrillar nucleoli present in some gonia (18). Continuous labeling with [^{3}H]TdR indicates the existence of a wide spread in the number of gonial cells synthesizing DNA in stage 53, ranging from 18 to 75% after 7 days of label. At this stage, gross differences between male and female gonads are too small to permit accurate sexing of tissues used in squashes. However, by the end of stage 53 in animals which are known phenotypic females (estradiol treated), intercellular bridges are found between at least some gonia (Fig. 2). This indicates that some of these cells have already reached the secondary gonial cell stage (Fig. 2) and presumably are committed to a defined number of divisions before entering meiotic prophase (26).

Results generally similar to those just described also apply to stage 54, which lasts from day no. 30 to day no. 36 of development, except that the percentage of labeled germ cells ranges from 25% to over 90% after 7 days of thymidine incorpora-

TABLE I

Differentiation and Thymidine-Labeling Kinetics during Early Germ Cell Development in Xenopus laevis

Day/Nieuwkoop-Faber stage at time of sacrifice	Maximum percent germ cells labeled after 1 wk of [^{3}H]TdR injection (See Materials and Methods)	Selected events in germ cell development
1/1	—	PGC in endoderm
4/43	—	PGC reach gonadal rudiment
7/47	0	1–2 nucleoli in PGC
10/48+	0	No changes in PGC
14/49	0	No changes in PGC
17/50+	0	No changes in PGC
20/51	0	No changes in PGC
21/51+	8	Overt sexual differentiation begins
22/52	30	First rDNA amplification and multiple nucleoli detected; first mitotic figures
25/53	42	
28/53+	75	First intercellular bridges in oogonia
35/54+	male/female	Sexes identifiable in squashes
	34/92	
40/56	54/96	First labeled premeiotic-S or leptotene in female
42/56+	48/100	
49/58+	65/96	Metamorphosis begins; first male premeiotic-S leptotene on day 52
56/63	76/92	
63/66	68/92	Metamorphosis complete
70/postmetamorphosis	64/88	
77/	73/94	
180/	62/10	Vitellogenesis in female, spermatogenesis in male

tion (Table I). Part of this spread probably reflects differences between male and female gonads, which still cannot be accurately identified in tissues used for squashes at this stage.

Stage 55, lasting from day no. 37 to day no. 41, is notable in several respects. First, by this time it is possible to identify unequivocally the sex of gonads for squashes. Second, in ovaries, the number of oogonia incorporating thymidine over a 7-day exposure period approaches 100%, while this figure remains considerably lower in testes (Table I). Finally, on or about day no. 40, the first cells in premeiotic S or early leptotene of meiotic prophase are observed in ovaries. Such cells are all labeled with thymidine, even when 3-day incorporation periods are used,[2] and represent less than 5% of the total ovarian germ cell population at this time. Thymidine labeling for shorter periods during this stage also provides further information about the replication of DNA during the gonial cell cycle. When cells are labeled for less than 24 h, nuclei are observed which preferentially label over nucleoli (Fig. 3). This pattern occurs in spermatogonia as well as in oogonia, and suggests that an asynchrony exists between chromosomal DNA synthesis and rDNA replication (27).

By stage 56, which extends from day no. 42 to day no. 45, the maximum percentage of female gonial cells labeled with [^{3}H]TdR after 7 days' continuous exposure is reached, representing 100% in at least some cases. All leptotene stage cells in the female at this stage are labeled, and do not exceed 10% of the total number of germ cells in the ovary. In the male, the percentage of labeled spermatogonia continues to increase slowly, but remains well below female levels (Table I). In contrast to the ovaries, the male gonads still contain only gonia at this stage.

At stage 57, which extends from day no. 46 to

[2] Since G_2 plus leptotene takes 4–5 days in female *Xenopus* (2, 27) all leptotene cells would always be labeled with an incorporation period exceeding the total duration of leptotene. If, as at day 40, all leptotene cells are labeled by shorter incorporation periods, then all such cells must have still been in premeiotic S when label was present.

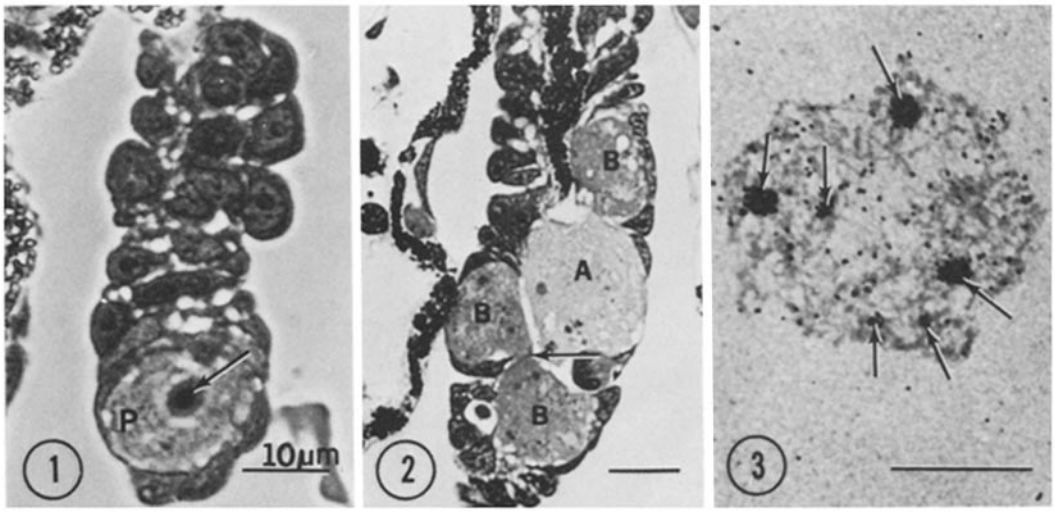


FIGURE 1 Phase-contrast light micrograph of a cross section of a stage 48 sexually undifferentiated gonadal rudiment. A large primordial germ cell (P) is visible with a single prominent nucleolus. (arrow). × 1,300.

FIGURE 2 Bright-field light micrograph of a longitudinally sectioned stage 53 ovary. A single primary oogonium (A) is flanked by smaller secondary oogonial cells (B). Electron microscope observations on adjacent thin sections indicate that the two lower secondary gonial cells are connected by an intercellular bridge located at arrow. × 800.

FIGURE 3 Autoradiographic squash preparation of a stage 55 (day 40) spermatogonial nucleus from an animal labeled in vivo with tritiated thymidine 24 h before sacrifice. The nucleoli (arrows) show a disproportionate amount of grains as compared to the chromosomal region, suggesting that nucleolar-associated DNA was preferentially replicated at the time of incorporation. × 1,800.

day no. 48, further changes in both male and female germ cell populations are observed. In the female, the first unlabeled leptotene cells are observed on day no. 46 when short (1–3-day), labeling periods are employed. Oogonial labeling continues to reach a maximum value of over 95% after 7 days of continuous label, but values of 100% can no longer be obtained, even when animals are given more frequent isotope injections. In the stage 57 male, the total percentage of spermatogonia labeled after 7 days continues to increase, but remains below female values (Table I). The next stage, stage 58, covers a period of only 24 h, during which no notable changes occur in the germ cells. This stage does, however, mark the onset of metamorphosis in the organism.

Stage 59, which extends through day no. 52, marks the appearance of the first labeled premeiotic S or leptotene cell in the testis. Again, as in the female, these meiotic cells constitute only a small fraction of the total germ cell population in the gonad, ranging from 2 to 5%. In the female, the percentage of meiotic prophase stages starts to rise, and some zygotene nuclei are now observed. By day no. 63 (stage 66), metamorphosis is almost complete, and a few pachytene nuclei with their characteristic cap of amplified rDNA are present within ovaries (14). In the male, while some leptotene and a rare zygotene cell are observed, there are still very few meiotic cells present. In the female, in contrast, as many as 50% of the germ cells are now starting prophase of meiosis.

By 6 mo of age, the first mature sperm are present in the testis, and a continuously renewing stem cell population is established (Table I). The ovary, in contrast, has undergone a wave of differentiation of oogonia into meiotic prophase stages, with the result that few oogonia remain in the ovary. Furthermore, the remaining oogonia appear to be largely dormant, with less than 10% incorporating [^{3}H]TdR (Table I). The ovary at this time contains approximately 3,000–6,000 oocytes

and only a few hundred oogonia. 25 primordial germ cells would each have to divide an average of 8–9 times to produce this number of cells.

In situ RNA-DNA Hybridization on Cytological Preparations of Germ Cell Nuclei

With the normal sequence of germ cell development established, the level of ribosomal DNA gene amplification in premeiotic germ cells was investigated using *in situ* RNA-DNA hybridization employing radioactive RNA complementary to amplified *Xenopus* oocyte rDNA (see Materials and Methods). At the relatively low levels of hybridization obtainable with nonamplified diploid cells, this technique is most accurate when comparisons of relative amounts of hybridization are made between closely adjacent nuclei on a single slide. Quantitation, however, is limited by two factors: first, differential spreading of a constant amount of DNA per unit area; and second, oversaturation of the emulsion at focal points of radioactivity. These variations result in deviations in grain count per unit of RNA actually bound. In terms of the present experiments, these variables would tend slightly to exaggerate the grain count observed over well-spread germ cell nuclei as compared to more compact somatic cell nuclei in the same preparation, making grain count variations within a factor of ± 0.5 nucleolus organizer equivalent not significant. This level of variation, however, represents only a small fraction of the total rDNA present in amplified premeiotic cells, since most gonia in newly metamorphosed animals contain from 5 to 40 times the normal diploid amount of rDNA (14) (see Discussion).

Within the relatively narrow half-organizer limit in variability, cytological hybridization results from the earliest stage examined, stage 43 (4 days), until stage 52 over 2 wk later, are similar in that no significant rDNA amplification could be detected in germ cells (Table II). Specific examples of this are illustrated in Figs. 4–11. Each germ cell nucleus characteristically shows one or two foci of hybridization with approximately the same total grain level as that observed over somatic cell nuclei in the same squash.

Between 22 and 24 days of development (Stage 52), the first clear increase in rDNA content of germ cells is detected by cytological hybridization. Several gonia are observed which have increased numbers of grains and/or increased foci of hybridization (Figs. 12–15). This increase is not present within all gonia from a single gonad, and the number of silver grains observed over different foci within a single gonial cell nucleus is often unequal. Since only a few germ cells at this stage have begun rDNA amplification, this initial increase is not clearly reflected in total grain counts (Table II), but is apparent in individual cells (Figs. 14, 15).

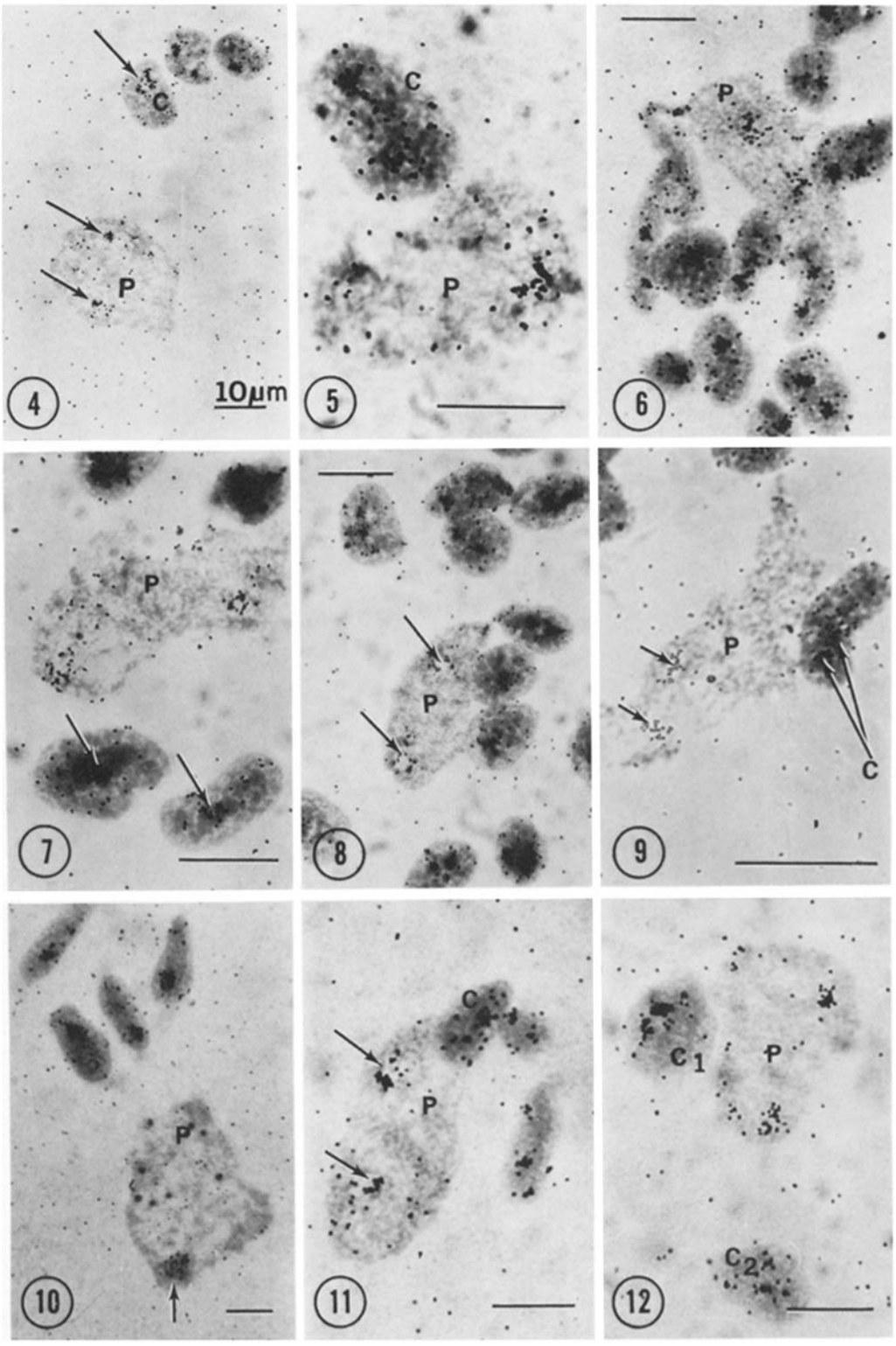
During the ensuing stages of premetamorphic development, a gradual increase occurs in both the number of amplified gonia and the level of amplification of rDNA within single cells in both male and female gonia[3] (Figs. 16–19). Hybridization levels observed in still later oogonial and spermatogonial stages indicate that rDNA amplification is present in gonia until the onset of meiotic prophase, when, as established by Pardue and Gall (22), the spermatogonia lose their extra rDNA. Cytological hybridization data obtained in the present investigation suggests that a reduction in the level of rDNA amplification probably occurs in female cells at the onset of meiotic prophase, even though rDNA amplification will start again in these cells in zygotene (2, 27). Grain counts over premeiotic S or leptotene nuclei in newly metamorphosed females indicate that approximately 12% of such cells have rDNA hybridization levels equal to somatic cell nuclei (Fig. 20, Table III).

Isolation of Satellite DNA from Adult Testes

Preliminary attempts have been made to study the nature of gonial rDNA amplification by more direct means. Meiotic oocyte amplified rDNA, which is unmethylated, has a greater density in neutral cesium chloride solutions than chromosomal rDNA, which contains methyl cytosine residues (10). If gonial amplified rDNA is also unmethylated, then it should be possible to detect its presence in DNA extracted from gonial cells.

This possibility was examined using DNA isolated from adult testes (see Materials and Methods). DNA was extracted from animals that had just been mated to reduce the number of nonamplified cells to a minimum, preventing over-

[3] Cytological hybridization indicates the maximum level of rDNA amplification reached by premetamorphic oogonia and spermatogonia is similar, equaling 20–40 nucleolus organizer equivalents.


C
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C1
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12

TABLE II

Comparison of Grain Counts between Primordial Germ Cell Nuclei and Somatic Cell Nuclei in In Situ Cytological RNA-DNA Hybrids

Stage/day	No. of determinations	Primordial germ cell : somatic cell average ratio
43/4	4	1.2 ± .4
47/7	5	1.4 ± .2
48/10	4	1.1 ± .3
50/18	7	1.3 ± .3
51/19	5	1.2 ± .3
*52/22-24	14	1.2 ± .2
*53/26	9	1.5 ± .3

For each determination, grain count per nucleus in a single primordial germ cell was compared to grain count per nucleus in the five nearest somatic cells on the slide. Each set of somatic counts was averaged and the standard deviation computed. Data for each stage was then averaged and the standard error of the means calculated to give the above figures.

* Includes some cells presumed to have begun rDNA amplification.

loading of the chromosomal rDNA peak in analytical cesium chloride gradients. Adult male gonads were used, since DNA extracted from even young premetamorphic ovaries could be contaminated by amplified rDNA from meiotic stages. Presumably, the only cells in the testis which might contain unmethylated rDNA cistrons are spermatogonia. The result of an analytical isopycnic density gradient centrifugation of the pooled heavy fraction testis DNA collected from an initial preparative CsCl density gradient run (see Materials and Methods) is shown in Figure 21 *a*. Four peaks of DNA are present: the lightest is *Xenopus* main peak DNA, at a density of 1.699 g/cm^3 (24); the second is an uncharacterized heavy satellite found in *Xenopus* with a density of 1.715 g/cm^3 (25); the third peak is chromosomal ribosomal DNA with a density of 1.724 g/cm^3 (5, 6, 12, 13, 25); finally, a small heavy peak is found at a density of 1.729 g/cm^3, the same density of amplified rDNA extracted from *Xenopus* ovaries (5, 6, 12, 13). Control gradients of liver DNA (Fig. 21 *b*) lack any detectable 1.729 satellite. Hybridization stud-

FIGURES 4–20 Autoradiographic squash preparations hybridized *in situ* with tritiated RNA complementary to amplified *Xenopus* rDNA as described in the text. P, primordial germ cell nucleus; C, somatic cell nucleus; S, spermatogonial nucleus; O, oogonial nucleus. All nuclei not lettered are from somatic gonadal cells.

FIGURE 4 Stage 47 PGC with two foci of hybridization containing fewer grains than foci observed over a nearby follicle cell (arrows). × 850.

FIGURE 5 Stage 48 PGC and its closest somatic neighbor with roughly equivalent grain counts. × 1,900.

FIGURE 6 Stage 50 PGC with two foci of hybridization surrounded by somatic nuclei showing typical fluctuations in "normal diploid" grain counts. × 1,200.

FIGURE 7 Stage 50 PGC showing well-spread foci of hybridization in comparison to those seen over somatic nuclei (arrows). × 1,600.

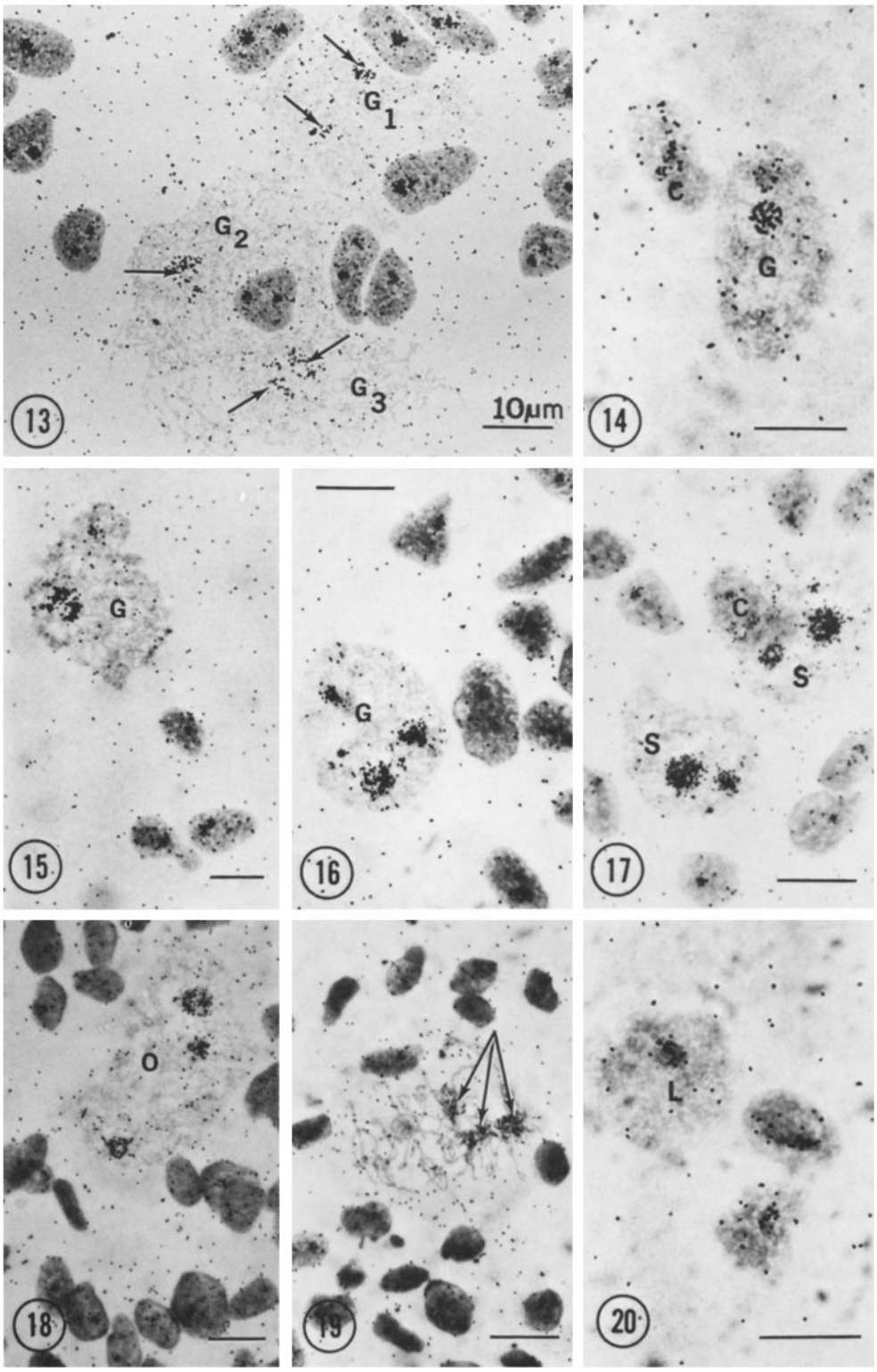
FIGURE 8 Stage 51 PGC with two foci of hybridization (arrows), and a typical distribution of somatic nuclei. × 1,200.

FIGURE 9 Stage 51 PGC and somatic cell nucleus, each with two approximately equal foci of hybridization (arrows). × 1,400.

FIGURE 10 Stage 51 PGC with a single focus of hybridization (arrow). × 800.

FIGURE 11 Stage 52 (day 21) PGC with two foci of hybridization (arrows) and a slight but not significant increase in grains compared to somatic nuclei. × 1,200.

FIGURE 12 Stage 52 (day 22) PGC showing two foci of hybridization and a grain density roughly equal to somatic nucleus C_1 but double the grain density of somatic nucleus C_2. × 1,400.


G1
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ies are now in progress to demonstrate conclusively that in fact the 1.729 satellite is rDNA.

DISCUSSION

The present investigation demonstrates that ribosomal DNA gene amplification in *Xenopus* is a two-step process, which first develops in the primary gonial cells of both sexes, and continues only in females during meiotic prophase. Within the limits of detection allowed by RNA-DNA cytological hybridization, no significant rDNA amplification is present in primordial germ cells. The first increase in the number of rDNA cistrons occurs over 2 wk after the PGC have become established in the gonad. This increase, in turn, occurs at approximately the same time as the beginning of mitosis and sexual differentiation of the gonia.

Since primordial germ cells do, however, average slightly higher grain counts than somatic cells in *in situ* cytological hybrids (Table II), it is not possible to state unequivocally that no extrachromosomal rDNA molecules exist during this stage. Their presence is considered unlikely, however, for a number of reasons. First, as stated previously, the observed minor discrepancy in grain count is to be expected as an artifact of preparation caused by better spreading of germ cell DNA as compared to somatic cell DNA. Second, the initial hybridization level observed in PGC remains constant up to stage 52, whereas changes are clearly detectable in later developmental stages. Third, the total variability in hybridization values between nominally equal primordial germ and somatic cells is minor, representing only a fraction of the rDNA present in a normal nonamplified cell. This is in marked contrast to the rDNA increases seen in gonia, which are between one and two orders of magnitude greater than the diploid rDNA value (Figs. 15–19) (22). Fourth, in addition to the RNA-DNA hybridization data, the fact that no thymidine incorporation occurs in PGC before stage 52 implies that no new rDNA synthesis occurs during this time. This contention is supported by the fact that it is possible to detect nucleolar labeling in later gonial stages with [^{3}H]TdR (Fig. 3), demonstrating that thymidine incorporation is sensitive enough to detect an amount of DNA on the order of a few nucleolus organizer equivalents. Finally,

FIGURE 13 Stage 52 (day 23) squash illustrating differential spreading of germ cell versus somatic cell nuclei. Three highly spread germ cell nuclei (G) are present with diffuse foci of hybridization and well-defined grains (arrows). In contrast, foci of hybridization over somatic nuclei are compact and have a high grain density indicating oversaturation of the emulsion. These conditions make comparison of hybridization levels between the two cell types impossible. × 1,000.

FIGURE 14 Stage 52 (day 23) gonial nucleus (G) with an increased level of hybridization compared to somatic nucleus (C). × 1,400.

FIGURE 15 Stage 52 (day 25) gonial cell nucleus (G) showing approximately a fivefold increase in hybridization compared to surrounding somatic nuclei. This amount of amplification represents the maximum observed at this stage. × 1,000.

FIGURE 16 Stage 54 (day 35) gonial nucleus (G) with multiple foci of hybridization totalling at least ten times the hybridization observed over nearby somatic nuclei. × 1,200.

FIGURE 17 Stage 57 (day 47) spermatogonial nuclei with highly amplified, unequal foci of hybridization. Total hybridization level is between one and two orders of magnitude greater than surrounding somatic nuclei. × 1,200.

FIGURE 18 Stage 59 (day 50) oogonial nucleus with multiple, highly amplified foci of hybridization. × 850.

FIGURE 19 Stage 59 (day 50) male *mitotic* prophase. The level of hybridization over the chromosomes (arrows) indicates a high degree of amplification in comparison to surrounding somatic nuclei. Therefore, this must represent a spermatogonial division. × 850.

FIGURE 20 Premeiotic S or leptotene nucleus (L) from a female just entering metamorphosis. The relative hybridization levels between germ and somatic nuclei do not appear to be significantly different. × 1,400.

TABLE III

Distribution of rDNA Amplification Levels in Female Premeiotic S-Leptotene Nuclei as Determined by In Situ Cytological RNA-DNA Hybridization

Germ cell: somatic cell average ratio	Total germ cells scored (n = 50)
	%
0.5–1.5	12
1.5–2.0	14
2.1–4.0	20
4.1–6.0	22
6.1–10.0	18
greater than 10.0	14

Each determination was made by comparing the number of grains localized over an individual germ cell nucleus to the average of the grains observed over the two closest somatic cell nuclei. Specimens used were from young toadlets 1–2 mo after metamorphosis.

it is unlikely that any conserved maternal extrachromosomal rDNA copies are present in offspring PGC, since it is known from genetic crosses (7) that amplified rDNA cistrons in offspring oocytes are derived from rDNA templates inherited in a nonmaternal, Mendelian manner.

The findings in the present investigation also allow several inferences to be made about the mitotic cycle of germ cells in the female. It is known that early prophase oocytes of *Xenopus* occur in nests of 16 cells (26). This means that germ cells must divide a minimum of four times before entering meiosis. It is also known that premeiotic S in *Xenopus* oocytes takes from 6 to 7 days (27). Labeling information from the present investigation may then be used to determine that the fastest-dividing cells in the maturing ovary undergo mitosis for a total of from 15 to 17 days from the time that they first synthesize DNA until the onset of premeiotic S. This would result in an average cycle time in the fastest-dividing cells from 3.8 to 4.3 days per division. This estimate is corroborated by the fact that in some stages, maximum labeling (greater than 95%) of gonia is observed within 5 days of daily labeling with [^{3}H]TdR. This cycle time is longer than the doubling time calculated for amplified rDNA by Watson Coggins and Gall (27), which is between 1.2 and 3.0 days per doubling.

Ribosomal DNA amplification persists in male and female gonial cells until the onset of meiotic prophase, when both gonial cell types appear to undergo a loss of rDNA at the onset of meiosis. The parallels in cytology and ultrastructure of these cells (18, 26), and the similarities in events occurring between the last gonial mitosis and leptotene, suggest that the initial phase of rDNA

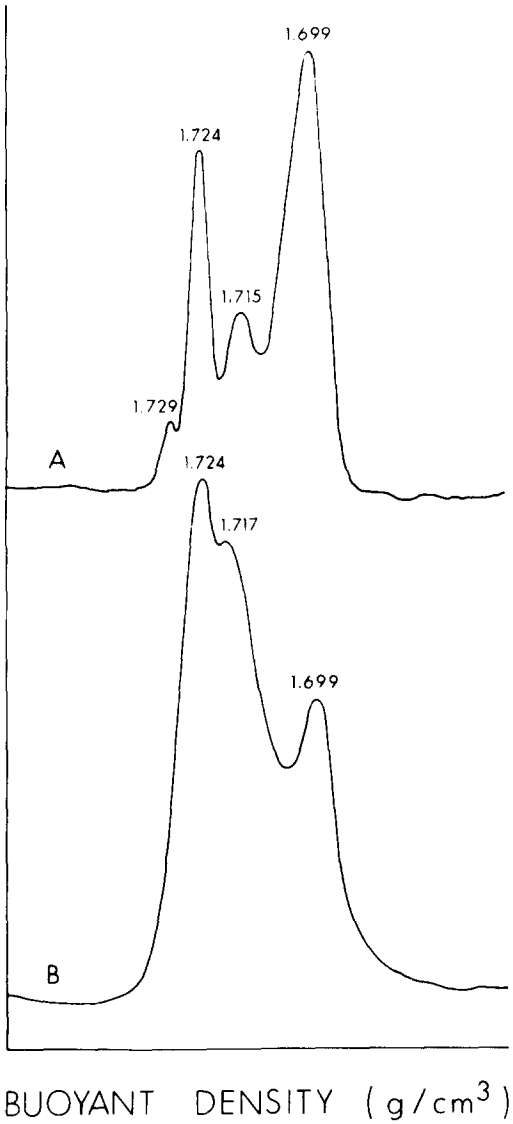

FIGURE 21 Analytical ultracentrifugation buoyant density profile of *X. laevis* "heavy fraction" DNA initially isolated by preparative cesium chloride isopycnic density gradient centrifugation (see text). (A) Testis DNA heavy fractions. (B) Liver DNA heavy fractions. Samples shown are from two different animals. Buoyant densities were calibrated by rerunning analytical gradients with added mouse satellite DNA, ρ = 1.690 g/cm^3.

amplification may be identical in both sexes. This hypothesis is strengthened by the fact that in cross-sex gonad transplants, meiotic stages which are initially present degenerate, but gonial cells remain viable and subsequently undergo the host sequence of germ cell differentiation and rDNA amplification in either reversal direction (unpublished observations).

The presence of a heavy satellite in *Xenopus* testis DNA corresponding in buoyant density to ovarian amplified rDNA lends further credence to the data obtained by *in situ* RNA-DNA hybridization on cytological preparations. The simplest explanation of the satellite is that it represents amplified rDNA derived from spermatogonia. Another possible explanation is that this satellite could be due to oocyte contamination from a hermaphroditic organ. Since one diplotene oocyte would equal from 100 to 1,000 gonial cells in amplified rDNA content, even minor contamination would be critical to this argument. To eliminate this possibility, extreme caution was used in selecting fully adult wild caught males known to be successfully bred. This precluded any possible stray laboratory hormonal influences (which may cause hermaphrodism in tadpoles) (16, 28) and in addition, provided animals whose fertility was tested before sacrifice. Furthermore, each testis was visually inspected for the possible presence of oocytes, and samples of each testis were squashed and examined microscopically for the presence of oocytes. In no case was any abnormal material found, so that a fair degree of certainty exists that the satellite is derived only from male tissue.

Observations on the behavior of oogonial stem cells suggest that there is some relationship between chromosomal DNA synthesis and amplified rDNA synthesis, at least in a temporal sense. As described earlier, after the initial wave of meiotic differentiation in the ovary, the remaining oogonia, which possess multiple nucleoli and a low level of rDNA amplification, become mitotically inactive. This withdrawal from the cell cycle normally lasts for one breeding period, a hiatus of from several months to a year (20). If amplification of rDNA in these cells continued independently of chromosomal DNA synthesis, they would soon accumulate massive caps of rDNA similar to those seen in pachytene oocytes. This is never observed, and furthermore, no rDNA increase can be detected by cytological RNA-DNA hybridization or thymidine incorporation.

Finally, the control of meiotic amplification must be exerted on an individual cellular level. Segmentally hermaphroditic genotypic males can be produced by estradiol pulse treatments (28). In such animals, both ovarian and testicular segments develop normal germ cells for several months, producing mature sperm and previtellogenic oocytes. In these cells, the pattern of rDNA amplication is totally normal in both cases. Thus, while a great deal of information exists about the timing and replication of ribosomal DNA amplification (3, 17), the factors which initiate and control this phenomenon remain to be elucidated.

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