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Differential Order of Replication of *Xenopus laevis* 5S RNA Genes

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In *Xenopus laevis* there are two multigene families of 5S RNA genes: the oocyte-type 5S RNA genes which are expressed only in oocytes and the somatic-type 5S RNA genes which are expressed throughout development. The *Xenopus* 5S RNA replication-expression model of Gottesfeld and Bloomer (Cell 28:781–791, 1982) and Wormington et al. (Cold Spring Harbor Symp. Quant. Biol. 47:879–884, 1983) predicts that the somatic-type 5S RNA genes replicate earlier in the cell cycle than do the oocyte-type genes. Hence, the somatic-type 5S RNA genes have a competitive advantage in binding the transcription factor TFIIIA in somatic cells and are thereby expressed to the exclusion of the oocyte-type genes. To test the replication-expression model, we determined the order of replication of the oocyte- and somatic-type 5S RNA genes. *Xenopus* cells were labeled with bromodeoxyuridine, stained for DNA content, and then sorted into fractions of S phase by using a fluorescence-activated cell sorter. The newly replicated DNA containing bromodeoxyuridine was separated from the lighter, unreplicated DNA by equilibrium centrifugation and was hybridized with DNA probes specific for the oocyte- and somatic-type 5S RNA genes. In this way we found that the somatic-type 5S RNA genes replicate early in S phase, whereas the oocyte-type 5S RNA genes replicate late in S phase, demonstrating a key aspect of the replication-expression model.

Frogs of the genus *Xenopus laevis* possess two families of genes that code for 5S rRNA (11). One family consists of some 20,000 oocyte-type genes that are expressed only in oocytes (22, 51). A second, smaller family of about 400 somatic-type 5S RNA genes is expressed in all somatic cells as well as in oocytes (22, 41). Genes within each family are essentially homogeneous in sequence (20), but oocyte-type and somatic-type genes have slightly different coding sequences and very different flanking sequences (9, 18, 19, 31, 36, 41; for a review, see references 28 and 30). We wish to understand how both families of genes are activated in oocytes and how the somatic-type genes are maintained in an active state in somatic tissues while the larger set of oocyte-type 5S RNA genes are inactive.

One approach to studying the differential regulation of the oocyte- and somatic-type 5S RNA genes is to use extracts of *Xenopus* oocytes that accurately transcribe the cloned 5S RNA genes in vitro (5, 9, 29, 37, 52). In vitro transcription of either class of 5S RNA genes requires a specific protein factor TFIIIA (16) in addition to the factors required by all genes transcribed by RNA polymerase III (32, 46, 49). TFIIIA, a protein of molecular weight 40,000, interacts with the intragenic control region of 5S RNA genes (6, 16, 42, 44). In the presence of the other factors necessary to form transcription complexes, this interaction is very stable (4, 7, 8, 32, 45, 47). If enough 5S DNA is added to a cellular extract to bind all the TFIIIA present, any 5S RNA genes added subsequently will not be transcribed.

It has also been determined that whereas TFIIIA is present in mature oocytes at about 3×10^{10} molecules per cell, or equivalently 5×10^5 molecules per 5S RNA gene, TFIIIA is present at less than one molecule per 5S RNA gene in somatic cells (39, 40, 48, 55). These facts led Gottesfeld and Bloomer (25), and Wormington et al. (56) to suggest a model for the regulation of 5S RNA genes that depends on the differential accessibility of oocyte-type and somatic-type genes to limiting amounts of TFIIIA in somatic cells. They propose that the somatic-type genes replicate earlier in the cell cycle than the oocyte-type genes and are thus able to bind all the available TFIIIA, so that only the somatic-type genes are transcribed. However, a key assumption of the model—that somatic-type genes in fact replicate earlier than oocyte-type genes—has not been tested. In this work, we used a fluorescence-activated cell sorter (FACS) to separate growing *Xenopus* cells into different fractions of the cell cycle. By analyzing each fraction, we determined that somatic-type 5S genes replicate early in S phase, whereas oocyte-type genes replicate late in S phase.

MATERIALS AND METHODS

Cell culture and labeling. *Xenopus laevis* kidney tissue culture cells (the generous gift of J. Gottesfeld) were grown at 21.5°C and 5% CO_2 in Gibco Glasgow minimal essential medium (G-MEM:BHK 21; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Irvine Scientific) and nonessential amino acids. Cells (30 to 50% confluent) used for analysis on the FACS were fed fresh media 24 h before labeling with bromodeoxyuridine (BUdR). Hypoxanthine (15 μg/ml), amethopterin (15 μg/ml), and BUdR (50 μM) were added to logarithmically growing cells 3, 5, or 40 h before harvesting as indicated in the Results section. In subsequent steps, exposure to all but yellow light was avoided.

Cell staining, flow cytometry, and sorting. BUdR-labeled cells were trypsinized from monolayers, fixed in 70% ethanol, treated with RNase, and stained with propidium iodide according to the method of Gray and Coffino (26).

Populations of cells corresponding to different phases of the cell cycle were sorted on the basis of DNA content in the FACS (FACS II; Becton Dickinson and Co., Mountain View, Calif.) by using a 590-nm-wavelength long pass filter with excitation at a 488-nm wavelength. Cells were separated into G1, four intervals of S phase, and G2-M subpopulations by setting the appropriate windows with respect to fluorescence intensity after scanning the exponentially growing population (see Fig. 2). The applicability of a FACS

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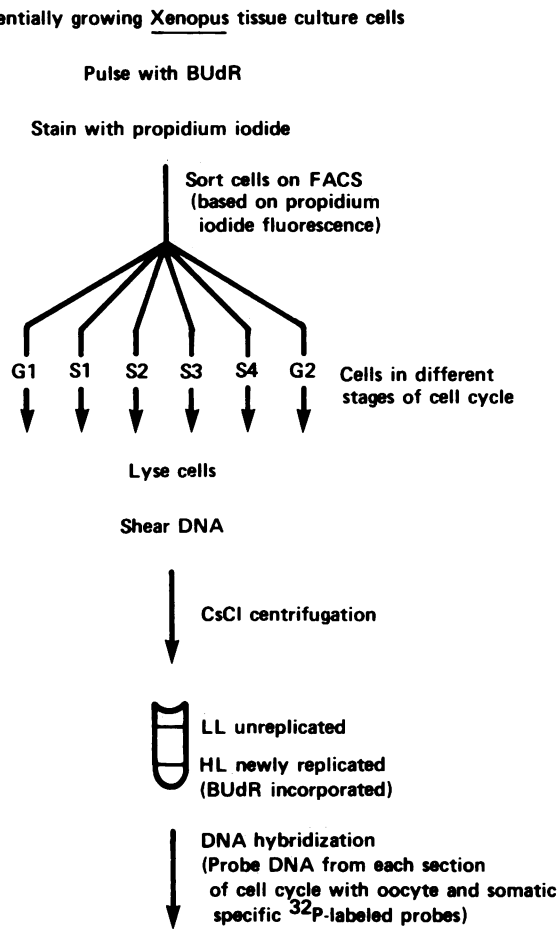

FIG. 1. Scheme for determining time of replication of the somatic-type and oocyte-type 5S RNA genes. Exponentially growing *Xenopus* tissue culture cells were pulsed with BUdR to density-label replicating DNA. The cells were fixed in ethanol, treated with RNase, and stained with propidium iodide. Cells from different stages in the cell cycle, and in particular from different portions of S phase, were separated on the FACS based on their propidium iodide fluorescence. The sorted cells were lysed, and their DNA was sheared. DNA was then separated into unreplicated (LL) and BUdR-substituted, replicated (HL) portions by CsCl buoyant density centrifugation. Fractions from each gradient were transferred to nitrocellulose by using a slot template. The filters were probed with either the oocyte-type or somatic-type ^{32}P-labeled probes (27) to determine the relative amounts of each gene which replicated in each portion of the cell cycle.

protocol for determining replication timing in *Xenopus* cells has been demonstrated by D. M. Gilbert (personal communication).

DNA isolation. Sorted populations of cells representing four sections of S phase in addition to G1, G2-M, and total S were incubated for 3 h at 55°C in DNA extraction buffer (0.01 M EDTA, 0.02 M Tris hydrochloride [pH 8.0], 50 μg of proteinase K per ml, 0.5% sodium dodecyl sulfate). Samples were adjusted to 0.2 M NaCl and sheared on ice by 10 passes through a 25-gauge needle and 5 passes through a 27-gauge needle by the method of Mariani and Schimke (35).

To separate newly replicated DNA (heavy-light [HL]) from unreplicated DNA (light-light [LL]), DNA samples from each portion of the cell cycle were dissolved in CsCl at a final density of 1.74 g/cm^3. Gradients were then centrifuged to equilibrium in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) at 125,000 × *g* in a Beckman Ti50 rotor for 42 to 68 h at 20°C. Thirty 0.42-ml fractions were collected from each gradient.

Gradient transfer and hybridization. The fractions from each CsCl gradient were denatured, neutralized, and transferred to nitrocellulose by using a slot template by the method of Brown et al. (12). The filters were then hybridized with ^{32}P-labeled nick-translated probes specific for either the somatic- or oocyte-type 5S RNA genes [pXls459 or pXlosp1 (27); in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 100 μg of denatured salmon sperm DNA per ml, 0.1% SDS, 1× Denhardt solution at 37°C by the method of Maniatis et al. (34)]. Washed filters were exposed to preflashed Kodak XRP X-ray film at −70°C and densitometric tracings of the autoradiograms were made by using a Helena Laboratories Quick Scan.

RESULTS

Experimental strategy. Our strategy for determining the times of replication of the oocyte- and somatic-type 5S RNA genes is outlined in Fig. 1. Exponentially growing *Xenopus* tissue culture cells were pulse-labeled with the thymidine analog BUdR. BUdR is incorporated into one strand of replicating DNA, increasing its density; the replicating DNA is therefore designated HL. DNA that does not replicate during the labeling period cannot incorporate BUdR in either strand and is hence designated LL.

The population of BUdR-labeled cells was stained with the DNA-binding dye propidium iodide. Cells that are more advanced in the cell cycle have more DNA and therefore stain more intensely, since propidium iodide fluorescence is proportional to DNA content (26). It was therefore possible to use a FACS to separate the cells into fractions that represented different cell cycle phases: G1, four successive subphases of the DNA replicating phase S, and G2 (Fig. 2).

DNA from the cells of each fraction was isolated and sheared. It was then dissolved in CsCl and centrifuged to equilibrium to separate the dense, newly replicated DNA (HL) from the unreplicated DNA (LL). The HL and LL DNA from each fraction of the cell cycle were separately hybridized with DNA probes specific for the oocyte- and somatic-type 5S RNA genes. If a probe specific for either class of 5S RNA genes hybridized significantly to the newly replicated DNA from a cell cycle fraction, that class of 5S RNA genes must have replicated during that part of the cell cycle.

Determination of replication parameters. To separate *Xenopus* cells into suitable cell cycle fractions, it was first necessary to determine the length of S phase in these cells. We began by determining the doubling time of the cells under our growth conditions. A simple growth curve showed that the doubling time was 40 h (data not shown). We next determined the percentage of cells in each phase of the cell cycle at a given time. Exponentially growing cells were stained with propidium iodide and analyzed on a FACS. Figure 2 is a histogram giving the relative number of cells with increasing propidium iodide fluorescence. The first peak represents cells in G1 phase (DNA content, 2.0) and contains 70% of the cells. The next region represents cells in S phase (DNA content between 2.0 and 4.0) and contains 23% of the cells. The final region, containing 7% of the cells, represents cells in G2-M (DNA content, 4.0). Since the number of cells in a phase at a given time is proportional to the length of that phase, the length of S phase can be calculated as 23% of 40 h or about 9 h.

To distinguish newly replicated DNA from unreplicated DNA after labeling with BUdR, it was necessary to determine the density of the BUdR-substituted HL DNA. A sample of *Xenopus* cells was grown in medium with BUdR for one generation. The base, hypoxanthine, and the folate analog, amethopterin, were also added to the medium to inhibit de novo synthesis of thymidine. DNA from cells that had been grown with or without the BUdR was centrifuged to equilibrium in cesium chloride. Fractions from each gradient were collected, and samples of each fraction were applied, in duplicate, to nitrocellulose by using a slot-blot apparatus (Fig. 3). One nitrocellulose blot was hybridized to a ^{32}P-labeled probe specific for oocyte-type 5S RNA genes, and the other blot was hybridized to a probe specific for somatic-type 5S RNA genes (27). The addition of BUdR led to a shift of about 10 fractions for oocyte-type 5S DNA, from a mean density of 1.6920 for LL DNA to a mean density of 1.7640 for HL DNA. The mean density of somatic-type 5S DNA shifted from 1.7100 for LL DNA to 1.7480 for HL DNA. The shift for somatic-type DNA was less than for oocyte-type DNA because somatic-type DNA has a much smaller A+T content than does oocyte-type DNA (37 versus 63%), so it incorporates less BUdR.

Time of replication of oocyte- and somatic-type 5S genes. An exponentially growing population of cells was labeled for 3 h with BUdR and then fixed and stained with propidium iodide. After a preliminary scan in the FACS (Fig. 2) the FACS windows were set to sort the cells into G1 phase, four consecutive parts of S phase, S1 through S4, and G2-M. DNA from each cell fraction was isolated, sheared, and centrifuged in CsCl. Gradient fractions were collected, applied to nitrocellulose, and hybridized to a probe specific for oocyte-type 5S RNA genes (Fig. 4A and B). DNA from G1 phase served as a marker for unreplicated LL DNA and corresponded to a mean density of 1.6920 for the oocyte-type 5S DNA. The position in the gradient of HL DNA was determined from its density of 1.7640 as measured in the experiment shown in Fig. 3. Hybridization of the probe to HL DNA of a cell cycle fraction meant that the oocyte-type genes were represented in the DNA replicating during that part of the cell cycle. Because the length of the labeling period (3 h) was long with respect to the length of one-fourth of S phase (2.25 h), DNA in each cell cycle fraction contained some DNA from the previous fraction. This explains why some DNA replication was seen in G2. Our results showed that there was no detectable replication of the oocyte-type genes in S1 and that almost all of the replication took place in the later parts, S3 and S4, of S phase (Fig. 4A and B).

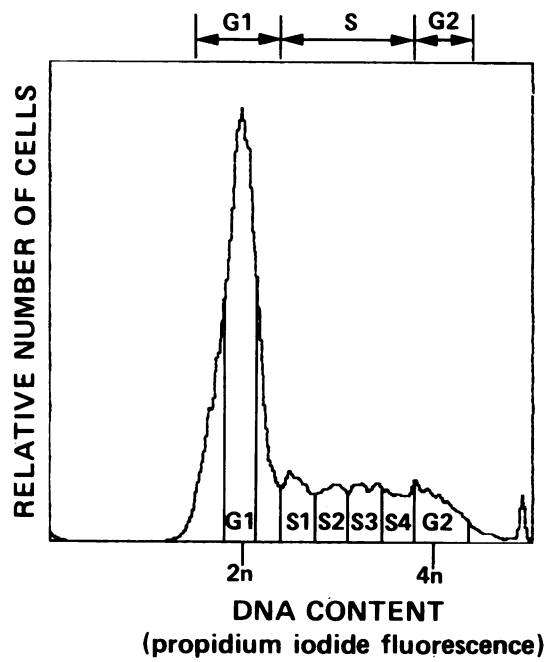

FIG. 2. Cell cycle profile of exponentially growing *Xenopus* tissue culture cells. The relative number of cells in each stage of the cell cycle is shown. Stages of the cell cycle are defined by DNA content, which is proportional to the propidium iodide fluorescence of the cells after fixation and treatment with RNase. The vertical lines within the profile indicate the gates set for each fraction of the cell cycle collected by the FACS (cells in G1 and G2-M and also cells in S phase, which were subdivided into four successive stages of S: S1, S2, S3, and S4).

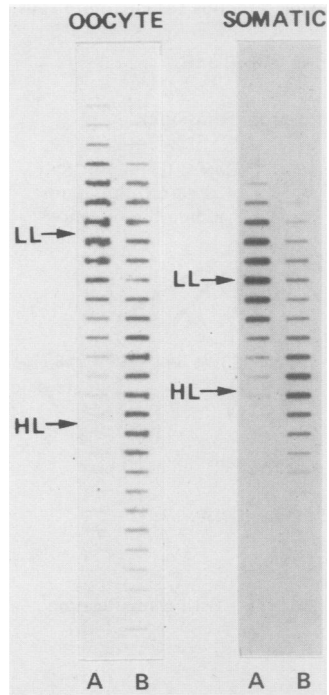

FIG. 3. Determination of the density of unsubstituted and fully BUdR-substituted oocyte-type and somatic-type 5S DNA. *Xenopus* tissue culture cells were grown in the absence (A) or presence (B) of BUdR to label replicating DNA. After one generation (40 h), the cells were harvested and lysed and the DNA was sheared. DNA was run on CsCl gradients, and each fraction of the gradient was applied to a slot in a slot-blot apparatus. The least dense fractions are shown at the top of the figure. Each blot was hybridized with either the oocyte-type or the somatic-type 5S-specific nick-translated probe. After one generation in BUdR, the density of the DNA shifted from LL to HL. For the 63% A+T-rich oocyte-type 5S DNA, these densities correspond to 1.6920 and 1.7640, and for the 37% A+T-rich somatic-type 5S DNA, these densities are 1.7100 and 1.7480, respectively.

In a similar experiment, *Xenopus* cells were labeled with BUdR for 5 h to increase the incorporation of BUdR and make it easier to separate the somatic-type HL DNA from the LL DNA. Slot blots of gradients containing DNA from the four fractions of S phase were then hybridized to a probe specific for somatic-type 5S RNA genes (Fig. 4C and D). Unreplicated DNA (LL) was defined by its mean buoyant density of 1.7100, and replicated DNA (HL) was defined by

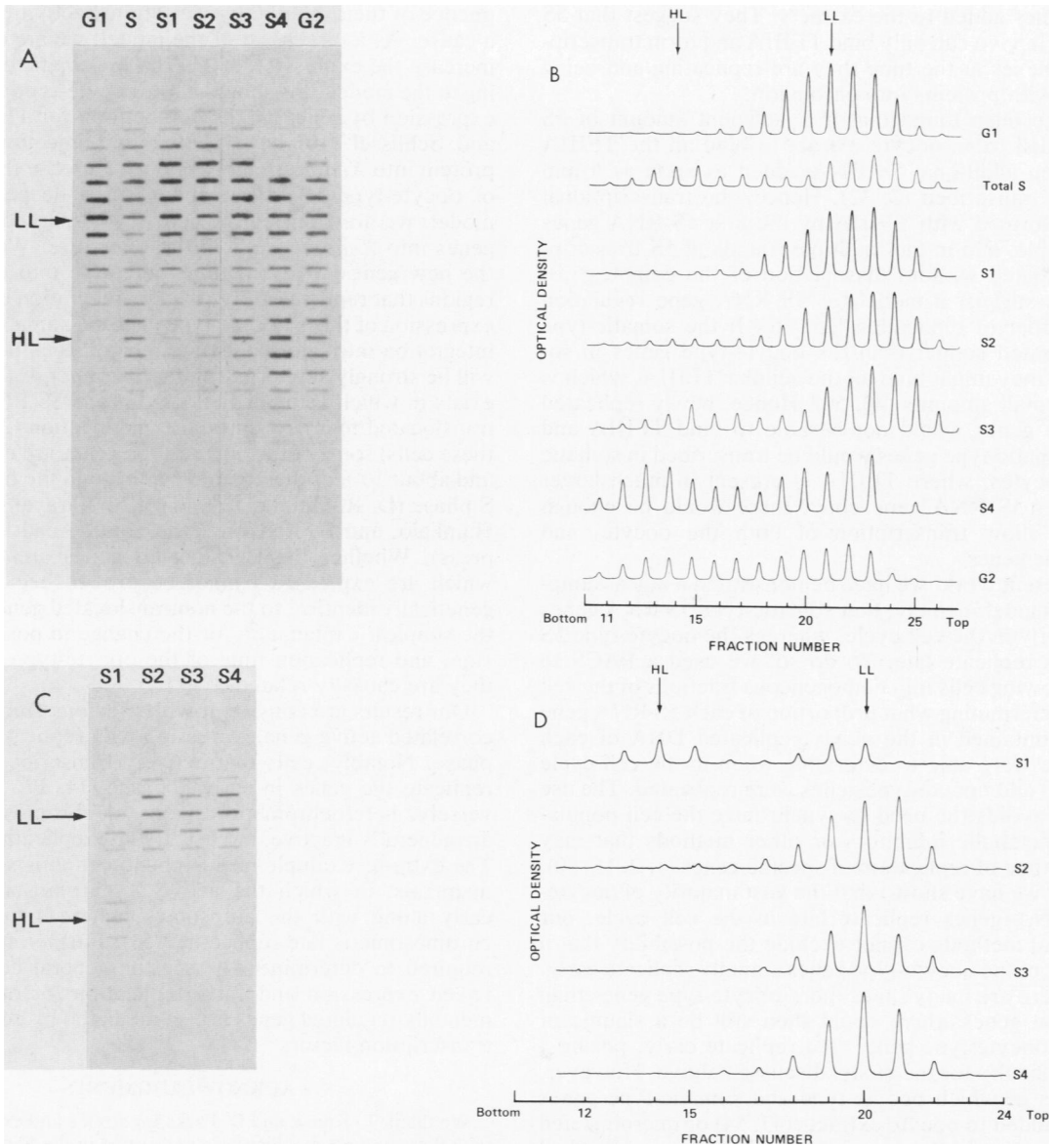

FIG. 4. Time of replication during S phase of the oocyte-type and somatic-type 5S RNA genes. *Xenopus* cells were pulse-labeled with BUdR and separated into portions of the cell cycle on the FACS. DNA from cells in each portion of the cell cycle (G1, S, and G2) and from the four quarters of the S phase (S1, S2, S3, S4) were run on CsCl gradients. Each fraction from the gradient was transferred to nitrocellulose by using a slot-blot apparatus. Lightest density fractions are at the top of the figure. Hybridization of a ^{32}P-labeled nick-translated probe specific for oocyte-type genes (A) or somatic-type genes (C) to slot blots of *Xenopus* DNA from each portion of the cell cycle is shown. LL shows the mean density of unreplicated oocyte-type (1.6920) and somatic-type (1.7100) 5S DNA, and HL shows the density of replicated oocyte-type (1.7640) and somatic-type (1.7480) 5S DNA. Densitometric tracings of the autoradiograms depicted in A and C are shown in (B) and (D), respectively.

its density of 1.7480. All of the somatic-type 5S DNA replication, as indicated by hybridization to HL DNA, took place in the early cell-cycle phases S1 and S2. In contrast to the results for oocyte-type 5S RNA genes, there was no apparent replication in the late phases S3 and S4. The results for both oocyte-type and somatic-type genes were reproduced in several similar additional experiments.

DISCUSSION

The genes encoding 5S ribosomal RNA in the genus *Xenopus* have been among the most studied developmentally regulated gene families (for a review, see references 8, 28, and 32). A fundamental question about these genes is how the somatic-type 5S RNA genes are kept active in all cell types while the larger class of oocyte-type 5S RNA genes is active only in oocytes. A valuable approach to this question has been to use extracts of oocytes that correctly transcribe cloned 5S RNA genes in vitro. 5S DNA can form two kinds of complexes with cellular proteins. In the presence of TFIIIA, an active transcriptional complex is formed (4, 7, 45, 47). In the absence of TFIIIA, an inactive DNA-protein complex is formed that cannot bind TFIIIA later. These results are true for both oocyte-type and somatic-type

5S RNA genes added to the extracts. They suggest that 5S RNA genes in vivo can only bind TFIIIA and form transcriptional complexes at the time they are replicating and being assembled with proteins into chromatin.

It has also been found that if a sufficient amount of 5S DNA is added to an oocyte extract to bind all the TFIIIA present, then additional 5S DNA added as soon as 5 min later is not transcribed (7, 32). Hence, the transcriptional complexes formed with TFIIIA by the first 5S RNA genes must be stable, and in fact multiple rounds of 5S transcription are initiated without dissociation of the complex (7). These facts suggest a model for 5S RNA gene regulation based on order of replication (25, 56). If the somatic-type genes replicated sooner than the oocyte-type genes in somatic cells, they might bind all the cellular TFIIIA, which is present in small amounts (40, 56). Hence, newly replicated oocyte-type genes would not be able to bind TFIIIA and only the somatic-type genes would be transcribed in somatic cells. In oocytes, where TFIIIA is present in much larger amounts per 5S RNA gene copy, there would be enough TFIIIA to allow transcription of both the oocyte- and somatic-type genes.

In the present work, we have demonstrated a key assumption of this model; namely, that somatic-type 5S RNA genes replicate early in the cell cycle, whereas the oocyte-type 5S RNA genes replicate late. To do so, we used a FACS to separate growing cells into homogeneous fractions of the cell cycle. By determining what proportion of each 5S RNA gene type was contained in the newly replicated DNA of each fraction, we were able to determine when in the cell cycle the somatic- and oocyte-type genes were replicated. The use of a FACS avoids the need to synchronize the cell population with metabolic inhibitors or other methods that may disturb the time of replication of specific genes (1, 2, 15, 50).

Although we have shown that the vast majority of oocyte-type 5S RNA genes replicate late in the cell cycle, our experimental methods cannot exclude the possibility that a few percent of the oocyte-type genes actually replicate early. Because there are many times more oocyte-type genes than somatic-type genes, there could then still be a significant number of oocyte-type genes that replicate early, posing a potential difficulty for the model discussed above. However, experiments in which oocyte-type and somatic-type genes both were added to oocyte extracts (43, 54) or microinjected into oocytes (L. J. Korn and J. B. Gurdon, unpublished results) have shown that the somatic-type genes are transcribed four times more efficiently than the oocyte-type genes and have a fourfold greater affinity for TFIIIA. Brown and Schlissel (10) have recently shown that the somatic-type 5S RNA genes are transcribed 25- to 200-fold more efficiently than are the oocyte-type 5S RNA genes when injected into cleaving *Xenopus* embryos. Even this dramatic difference in transcription efficiency does not fully account for the total quiescence of the large oocyte-type 5S RNA gene family in somatic cells. Perhaps the magnitude of this in vivo preference together with regulation via replication order can account for the differential expression of the 5S RNA genes. That is, even if a limited number of oocyte-type genes replicated early, the somatic-type genes might have a sufficient competitive advantage to effectively exclude transcription of the oocyte-type genes.

Although the results presented here, in combination with previous findings, lend support to the replication-expression model for 5S RNA gene regulation, they are not sufficient to prove it. For example, the order of replication of the oocyte- and somatic-type 5S RNA gene families might be a consequence of their relative transcriptional activities rather than a cause. As a direct test of the model, we are attempting to increase the expression of TFIIIA in somatic cells. According to the model, this should allow oocyte-type 5S RNA gene expression by relieving the competition for TFIIIA. Brown and Schlissel (10) have shown that injection of TFIIIA protein into *Xenopus* embryos does increase the expression of oocyte-type 5S RNA genes. For a deeper test of the model, we are transfecting genetically marked oocyte-type genes into *Xenopus* cells in tissue culture. We expect that the new genes will sometimes integrate into chromosomal regions that replicate early. If such integration events lead to expression of these oocyte-type genes in somatic cells, while integration into late-replicating regions does not, the model will be strongly supported. In fact, a *Xenopus laevis* cell line exists in which some of the oocyte-type 5S RNA genes are translocated to a new chromosomal location (27 and 38). In these cells, some of the oocyte-type genes are expressed (21) and about 10% of these genes replicate in the first quarter of S phase (D. R. Guinta, J. Y. Tso, S. Narayanswami, B. A. Hamkalo, and L. J. Korn, Proc. Natl. Acad. Sci. USA, in press). Whether the translocated genes are indeed those which are expressed cannot be proven because they are genetically identical to the nontranslocated genes. However, the simplest explanation for the change in position, expression, and replication time of the oocyte-type genes is that they are causally related.

Our results are consistent with previous studies that have correlated active gene expression with replication early in S phase. Notably, cells that actively transcribe globin genes replicate the genes in early S phase (14, 17, 23, 24). Conversely, heterochromatic regions of chromosomes, which are generally inactive, are usually late replicating (3, 13, 33). The extreme example of this phenomenon is seen in female mammals, in which the active X chromosome replicates early along with the autosomes, whereas the inactive X chromosome is late replicating (53). Further studies will be required to determine whether the general correlation between expression and early replication for other developmentally regulated genes is also mediated by competition for transcription factors.

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ADDENDUM

The temporal order of replication of the *Xenopus* 5S RNA genes has been demonstrated by D. M. Gilbert as well (D. M. Gilbert, Proc. Natl. Acad. Sci. USA **83**:2924–2928, 1986).

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