Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2924–2928, May 1986
Cell Biology

Temporal order of replication of *Xenopus laevis* 5S ribosomal RNA genes in somatic cells

(cell cycle/cell sorter/gene expression/transcription)

DAVID M. GILBERT

Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305

Communicated by Stanley N. Cohen, December 31, 1985

ABSTRACT **The timing of replication of both the somatic and oocyte-type 5S ribosomal RNA genes of *Xenopus laevis* was determined in cultured cells by using 5-bromodeoxyuridine labeling of DNA coupled with a retroactive synchrony technique employing the fluorescence-activated cell sorter (FACS). The somatic genes replicate very early in S phase, while the oocyte genes replicate very late. These experiments provide direct evidence for a model [Gottesfeld, J. & Bloomer, L. S. (1982) *Cell* 28, 781–791] in which the transcription-activating factor TFIIIA is required at the time of *Xenopus* 5S rRNA gene replication to facilitate transcription of these genes.**

Eukaryotic DNA replication proceeds according to a specific temporal order (for reviews, see refs. 1–3). Although the biological significance of this precisely regulated cellular process is unknown, several lines of evidence suggest that early replication may be necessary (albeit not sufficient) for the establishment of a transcriptionally active chromatin configuration. For example, heterochromatin, considered to be transcriptionally inactive, is late-replicating, whereas the active euchromatin replicates early (4). Active genes that have been translocated into heterochromatic regions in certain cell lines have been shown to become inactive (5). In addition, the active X chromosome in female mammals is early-replicating, while the inactive X is late-replicating (6); when the inactive X is reactivated in differentiated cells, either by fusion with teratocarcinoma cells (7) or by treatment with inhibitors of DNA methylation (8), a coincident switch of the timing of replication of that chromosome to early S phase has been observed. Finally, examination of specific gene sequences has revealed that, if a gene is transcriptionally active, it is replicated early in S; genes that are inactive may be either early- or late-replicating (reviewed in ref. 9).

A possible mechanism by which early replication might comprise a necessary condition for gene expression has come from studies done with the 5S ribosomal RNA genes (5S genes) of *Xenopus laevis*. Two sets of *Xenopus* 5S genes exist: the somatic type is active throughout development; the oocyte-type is active only in developing oocytes and mid-blastula embryos. Initiation of transcription of both gene families depends on the availability of the 5S-specific transcription factor, TFIIIA, which binds to an intragenic control region shared by both gene types (10). Gottesfeld and Bloomer (11) have shown that this transcription factor is required prior to the assembly of nucleosomes for the formation of stably active transcription complexes *in vitro*, and that transcription complexes formed in the absence of TFIIIA or in the presence of an excess of histones are stably repressed and are refractory to the subsequent addition of TFIIIA. Based on these results, they proposed a model by which TFIIIA is required at the time of 5S gene replication to mediate the formation of an active chromatin configuration. The model predicts that if the somatic genes are replicated prior to replication of oocyte genes in somatic cells, then the somatic genes would have a competitive advantage in forming stable transcription complexes with TFIIIA, which is present in limiting amounts in somatic cells (12, 13) and has been shown to be stably associated with somatic genes but not oocyte genes in somatic cell chromatin (13). The formation of such complexes would lower the titer of diffusable TFIIIA in the cell, making it unavailable to the later-replicating oocyte genes.

I have determined the timing of replication of the oocyte and somatic type 5S genes in *X. laevis* tissue culture cells which have been shown to express exclusively somatic-type 5S RNA (14). A number of approaches have been applied to study the timing of replication of DNA sequences (15–17). A rapid and simple retroactive synchrony technique has been devised (D.M.G. and S. N. Cohen, unpublished work) that avoids the use of cumbersome prospective synchrony techniques and their accompanying artifacts. Using this procedure, I have found that the somatic genes are, indeed, replicated prior to the oocyte genes, as the Gottesfeld–Bloomer model predicts; the somatic genes are replicated very early in S phase, whereas the oocyte genes are replicated very late in S.

MATERIALS AND METHODS

Cell Culture and Labeling. *X. laevis* tissue culture cells were the gift of J. Gottesfeld. Cells were maintained in tightly sealed flasks, gassed with a 5% CO_2/95% air mixture, in a temperature-controlled room at 21.5°C. For labeling with 5-bromo-2′-deoxyuridine (BrdUrd), confluent cultures were split 1:5 and 2 days later incubated for 3 or 4 hr with BrdUrd (50 μg/ml), 5-fluoro-2′-deoxyuridine (5 μg/ml), and deoxycytidine (10 μg/ml).

Fluorescence-Activated Cell Sorter (FACS) Analysis and Sorting of Cells. At the end of the metabolic labeling, cells were trypsinized and stained with chromomycin A3 for FACS analysis by the procedure of Gray and Coffino (18). Analysis and sorting of cells was done using a FACS II (Becton Dickinson) with a 495-nm long-pass filter. Cell suspensions (10^7 cells per ml) were sorted at a flow rate of 4000 cells per sec and collected into siliconized glass tubes. To check the fidelity of sorting, 50 μl of sorted cells were added to 150 μl of chromomycin staining solution and, after 30 min at room temperature, were reanalyzed on the FACS.

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Abbreviations: BrdUrd, 5-bromo-2′-deoxyuridine; FACS, fluorescence-activated cell sorter; HL, heavy·light; LL, light·light; 5S gene(s), 5S rRNA gene(s).

Separation of Replicated and Unreplicated DNA. Cells from each FACS collection tube were transferred to Eppendorf tubes, centrifuged, resuspended in 150 μl of DNA extraction buffer (1% $NaDodSO_4$/100 mM Tris Cl, pH 8.0/200 mM EDTA with proteinase K at 100 μg/ml), and incubated at 56°C for at least 2 hr. Samples were then extracted once with phenol, once with phenol/chloroform (1:1 vol/vol), and once with chloroform; ethanol-precipitated, and resuspended in 10 mM Tris Cl, pH 8.0/1 mM EDTA with DNase-free RNase A at 10 μg/ml. When the RNA was completely digested, samples were loaded on $CsSO_4$ gradients in 5.2-ml centrifuge tubes, the refractive index was adjusted to 1.3705, and gradients were centrifuged at 30,000 rpm in a VTi80 (Beckman) rotor for at least 48 hr. Gradients were then collected from the bottom into 28 equal-volume fractions, using a peristaltic pump.

Hybridization and Quantitation. Aliquots of the DNA in $CsSO_4$ solution, from fractions 4–27 of the gradients, were denatured with NaOH and applied to nitrocellulose filters using a slot blot apparatus according to the procedure of Brown *et al.* (19). Plasmids carrying *X. laevis* somatic and oocyte 5S gene spacer probes (20) were the gift of L. Korn. The probes used in these experiments were prepared by standard nick-translation procedures and checked for probe specificity by hybridization to a Southern blot of *X. laevis* DNA, a cloned 5S gene repeat, and mouse DNA. Quantitation of the amount of somatic and oocyte spacer sequences present in each fraction of the gradients was performed using a Helena Laboratories Quick Scan R&D densitometer.

RESULTS

The results presented here were acquired in two separate experiments involving determination of the timing of replication of both the somatic and oocyte 5S genes within the same experiment. The complete data obtained in one of those experiments and the final data from both are shown. Five additional experiments in which data were separately acquired for either the oocyte or the somatic genes gave results similar to those presented here.

Retroactive Synchrony of Cells Using the FACS. *X. laevis* tissue culture cells were labeled for 3 or 4 hr with BrdUrd and then stained with the DNA-specific dye chromomycin to obtain the DNA histogram shown in Fig. 1*A*. Since *Xenopus* tissue culture cells are tetraploid, the G_1 and G_2/M peaks represent cells with a 4*N* and 8*N* DNA content, respectively. Populations of cells in different stages of the cell cycle were sorted by choosing the windows shown in Fig. 1*A*, each of which represents cells having a similar DNA content. To confirm the purity of the fractions, 1–3 × 10^5 cells were collected per window and 10^4 cells from each sample were restained with chromomycin and reanalyzed (Fig. 1*B*).

Replication Patterns of Oocyte and Somatic 5S Gene Sequences. To quantitate the fraction of replicated oocyte and somatic 5S gene sequences in each of the FACS-synchronized populations, BrdUrd-substituted (heavy·light, HL) DNA was separated from unsubstituted (LL) DNA by $CsSO_4$ gradient centrifugation. DNA from each fraction collected from the gradients was immobilized on nitrocellulose by use of a slot blot apparatus and hybridized to ^{32}P-labeled oocyte and somatic gene spacer probes that had been prepared by nick-translation and checked for specificity by Southern blotting. Oocyte and somatic 5S gene sequences share 97% homology but are organized as separate clusters having different spacer sequences (21). Fragments of these spacer sequences have been subcloned from cloned 5S repeats of each type and have been used to show the chromosomal locations of the two gene types (20). As these experiments utilized a slot blot apparatus for analyzing density gradient fractions and there are no distinguishing features such as restriction fragment size to confirm the integrity of the probe, it was important to ensure that each probe was hybridizing only to the appropriate sequences on the blot. For this reason the specificity of each nick-translation was checked by probing Southern blots with aliquots of the same probe to be used with the slot blots (data not shown).

[In the process of carrying out the above analysis, I found

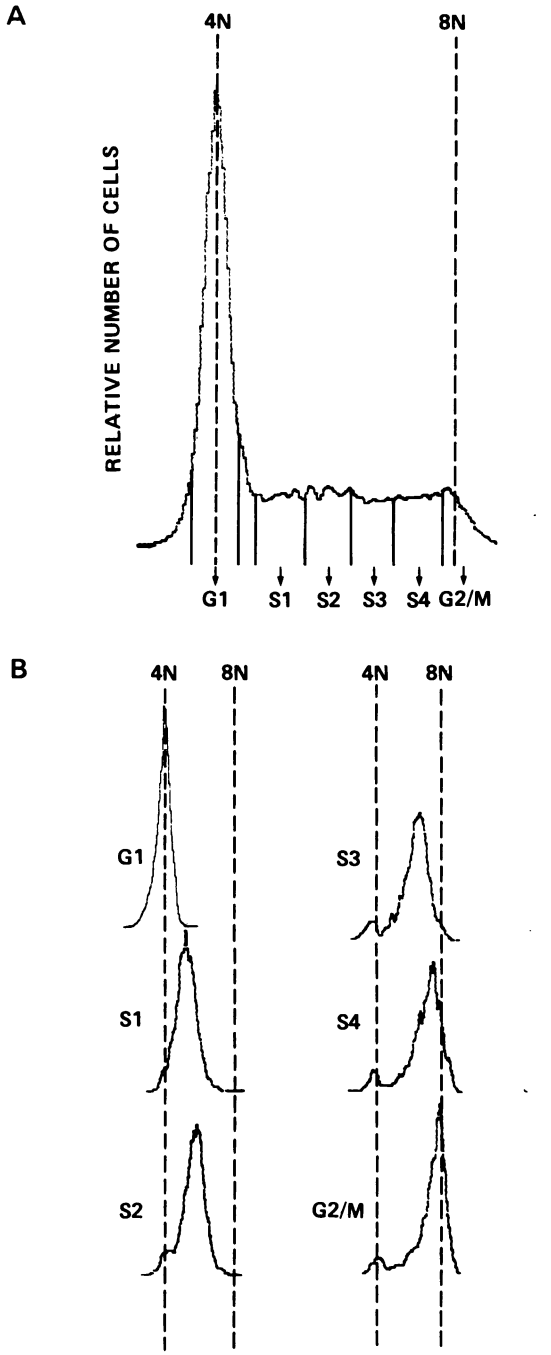

FIG. 1. (*A*) DNA histogram of logarithmically growing *X. laevis* tissue culture cells labeled for 4 hr with BrdUrd. The relative number of cells is plotted on the ordinate and DNA content on the abscissa; *N* is the haploid DNA content of the *Xenopus* cells, which are tetraploid, or 4*N*. The windows chosen for sorting synchronized populations of cells in different stages of the cell cycle are shown. S1–S4, sections 1–4 of S phase, respectively. (*B*) Analysis of the fractions sorted from the windows shown in *A*, demonstrating the degree of enrichment for populations with increasing modal DNA content.

a restriction-site polymorphism in the somatic genes of the *X. laevis* tissue culture line, manifested as a cytidine rather than a guanosine at a site 422 base pairs from the originally reported *Hin*dIII site, giving an additional *Hin*dIII site (data not shown). An interesting point about this polymorphism is that my data indicate it has spread to all 400 copies of the tandemly repeated somatic 5S genes.]

The results of hybridization of the immobilized HL and LL DNA are shown in Fig. 2 *A* and *B*. Each slot-blot lane represents the fractions from a single gradient. The absence of HL sequences in material isolated from G_1 cells and analyzed with either the somatic (Fig. 2*A*) or oocyte (Fig. 2*B*) probe provides a further control indicating that the cell-cycle fractionations were successful. In the first section of S phase, most of the somatic gene sequences are in the HL DNA; however, no significant replication of the oocyte genes is seen and all the DNA that hybridizes with the oocyte probe is at the LL density. Toward the end of S phase, however, replication of somatic genes no longer occurs, whereas the replication of oocyte sequences is greatest in the fourth section of S phase. Since cells labeled with BrdUrd for 4 hr were retroactively synchronized by selection of populations through a time window, some cells in G_2/M were at the end of S phase during the BrdUrd pulse. Hybridization was seen for the oocyte-type genes in the HL DNA of G_2/M, indicating that these genes replicate very late in S. No HL somatic gene DNA was found in this time period.

Fig. 3 shows the extent of replication of the two gene types in each section of the cell cycle as the percentage of the total HL plus LL hybridization signal per gradient that was represented in the HL fractions. The results of two separate experiments are shown. In one, cells were labeled with BrdUrd for 3 hr (Fig. 3*A*) and in the other, for 4 hr (Fig. 3*B*). The data presented in Figs. 1 and 2 were obtained in the experiment of Fig. 3*B*. Both experiments show the same result; somatic 5S gene replication occurs primarily in early S phase, whereas oocyte 5S gene replication occurs late in S.

DISCUSSION

The results presented here show that, in *X. laevis* somatic cells, somatic 5S rRNA genes are replicated prior to oocyte-type 5S rRNA genes. Moreover, the somatic-type genes are replicated very early and the oocyte genes very late, giving the somatic genes the maximum competitive advantage available to them by this means for binding to limiting amounts of 5S-specific transcription factor, TFIIIA. These findings can account for the expression of the somatic genes to the exclusion of the 50-fold more numerous oocyte genes in somatic cells (14) and provide direct support for the model proposed in 1982 by Gottesfeld and Bloomer (11). The model requires that TFIIIA be available for DNA interaction at the time of 5S gene replication and therefore predicts that some cell-cycle control be exerted over the TFIIIA molecule to limit its availability in somatic cells to early S phase, when the somatic genes are replicated. A final demonstration of the correctness of the model would be to show that TFIIIA is available in the nucleus for binding to the somatic genes in early S phase, when the somatic genes are replicated, and not late in S, when the oocyte genes are replicated.

The model described by Gottesfeld and Bloomer was invoked to explain the control of 5S gene expression in somatic cells. However, it also suggests possibilities for how the activation of oocyte genes occurs in the oocyte. The

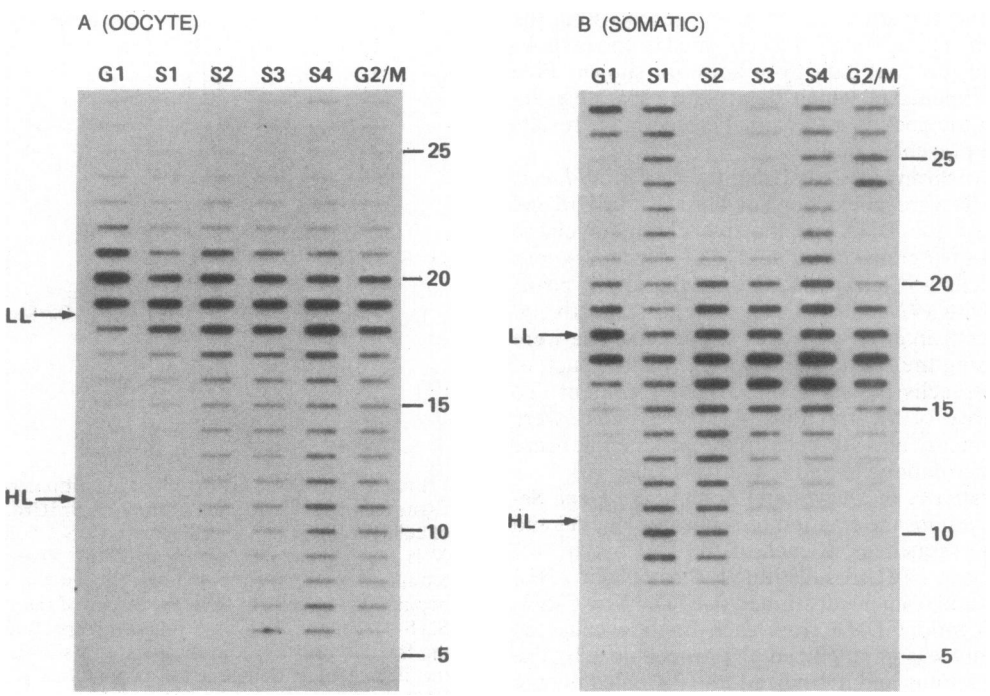

FIG. 2. Separation of HL and LL DNA by density equilibrium centrifugation in $CsSO_4$ gradients. Twenty-eight fractions were collected from the bottom of each gradient and aliquots of fractions 4–27 were applied to nitrocellulose filters by use of a slot blot apparatus and were hybridized with oocyte- (*A*) or somatic- (*B*) specific spacer probes, which were simultaneously checked for specificity by probing Southern blots of *Xenopus* DNA. Each lane represents the fractions (numbers at right) from a single gradient adjusted so that the center of the gradient was at a density between that of LL and HL *X. laevis* genomic DNA. The locations of HL and LL DNA are indicated, and the cell-cycle stage from which the DNA was isolated is indicated above each lane. The bands of hybridizing material seen at the very top of the gradient were occasionally observed in DNA prepared from all sources, including plasmids isolated from *Escherichia coli*, and do not appear to be enriched for either replicated or nonreplicated DNA. They appear at the same intensity for both oocyte and somatic gene probes, when the exposure time for both is equivalent, and are presumably due to the presence of protein contaminants in the gradients. They were not seen in either of the gradients of experiment 1 (see legend to Fig. 3).

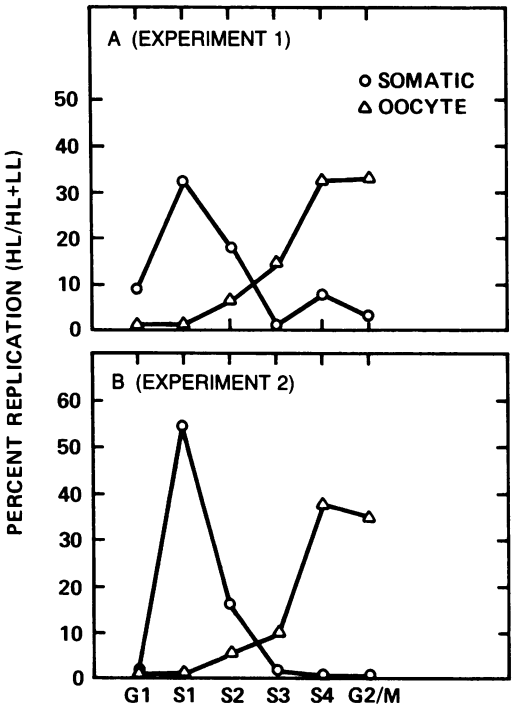

FIG. 3. Temporal order of replication of the oocyte and somatic 5S genes. Data are from two separate experiments. Cells were metabolically labeled with BrdUrd for 3 hr (*A*, experiment 1) or 4 hr (*B*, experiment 2) and then were retroactively synchronized with the FACS. The percentage of each of the two types of sequence replicated during the pulse for each stage of the cell cycle was determined by slot blot hybridization analysis. Percent replication is defined as the percentage of the specific sequence replicated (present in HL DNA) per total (HL + LL) DNA from each cell population. Points plotted at the very bottom of the graph indicate no HL DNA was detected for that gradient, at a sensitivity of <1%. The small amount of somatic HL sequences seen in G_1 of experiment 1 but not experiment 2 is probably due to contamination of the G_1 fraction with early S-phase cells, for cell-cycle analyses indicated less uniform chromomycin staining of cells in experiment 1. Hybridization to the HL DNA of S4 and G_2/M for the somatic genes is seen for experiment 1 but not experiment 2, presumably because of the observed presence of doublets of cells, in early S phase of experiment 1 but not experiment 2, that fluoresced as late-S-phase cells and were sorted into the late-S windows, rather than because of true replication of some of the somatic genes in late S phase of experiment 1 but not of experiment 2.

overproduction of TFIIIA in the oocyte, to over 10^6 times the level found in somatic cells (12, 13), potentially can account for activation of the oocyte-type genes in oocytes by relieving the competition of the two gene types for the positively acting TFIIIA. It is also possible that the time of replication of the oocyte 5S genes relative to the somatic 5S genes could be altered in the *Xenopus* oocyte, which could relieve the competition for TFIIIA and bring about oocyte 5S gene expression. The current experiments have not addressed the timing of replication of the oocyte- and somatic-type genes in oocytes.

Brown and Schlissel (14) recently have found, by coinjection of oocyte and somatic cloned genes into *Xenopus* embryos, that somatic genes are transcribed 100- to 400-fold more efficiently than oocyte genes; they also found that by microinjecting large amounts of TFIIIA into *Xenopus* embryos, they could activate oocyte RNA synthesis even in the absence of DNA replication. This finding, coupled with the earlier result that histone H1 seems to mediate the stable repression of oocyte 5S genes in somatic cells (22), suggests that histone H1 and TFIIIA are exchangeable *in vivo*, and that the differential expression of the two gene types in somatic cells might be explained simply by the affinities of the respective control regions for TFIIIA and by the nuclear concentration of TFIIIA relative to that of the repressing protein histone H1. While such an interpretation is seemingly contradictory to the Gottesfeld–Bloomer model, the two views are not mutually exclusive. The observed 100- to 400-fold preference for somatic gene transcription in embryos yields an absolute level of somatic gene transcription only a few times higher than that of the oocyte genes, since there are 50 times as many oocyte genes as somatic genes, and, thus, still does not explain the exclusive production of somatic 5S RNA in somatic cells. It could be that the competitive advantage provided to the somatic genes through early replication ensures their exclusive transcription. Also, it may be that in the developing embryo, conditions are more favorable for the exchange of histone H1 with transcription factors than in differentiated somatic cells. It would be of interest to determine the effect of an overabundance of TFIIIA, in the absence of DNA replication, on the transcription of oocyte-type genes in *Xenopus* tissue culture cells.

The model for the expression of the *Xenopus* 5S genes investigated in this report also suggests a possible role for the temporal order of DNA replication in regulating the expression of other eukaryotic genes. Potentially, the replication of a gene at the time when a functional excess of activating factor(s) is available is a necessary requirement to form an active chromatin conformation. The transcriptional potential of genes competing for common activating factor(s) could then be regulated by altering the level of activating factor or by a change in the timing of replication of the genes. It is not known whether the temporal order of replication of eukaryotic genes is the same in different tissue types, and I know of no direct evidence for a mechanism to alter the replication time of genes during development. However, the results presented here suggest the need for further studies to address this question.

My special thanks go to Stanley Cohen for discussions, advice regarding experimental protocols, financial support through National Institutes of Health Grant GM27241, and use of laboratory space, as well as for comments and suggestions on the manuscript. I thank Charles Omer for invaluable discussions throughout the project, Brian Mariani for pointers and needed references, and Diane Guinta for discussions and for first showing that DNA could be isolated from ethanol-fixed cells. Thanks also to David Parks for sharing his knowledge of the FACS and for expert technical assistance in the early stages of the project. During the work, I was supported by a predoctoral fellowship from National Institutes of Health Training Program for Ph.D. Candidates GM07790.

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