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Early replication and expression of oocyte-type 5S RNA genes in a *Xenopus* somatic cell line carrying a translocation

(developmental regulation/replication order/chromosomal translocation)

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ABSTRACT **In *Xenopus* somatic cells, the somatic-type 5S RNA genes replicate early in S phase, bind the transcription factor TFIIIA, and are expressed; in contrast, the late replicating oocyte-type genes do not bind TFIIIA and are transcriptionally inactive. These facts support a model in which the order of replication of the somatic-type versus the oocyte-type 5S genes causes their differential expression in somatic cells due to sequestration of TFIIIA by the early-replicating somatic genes. Here we provide further evidence for the model by showing that in one *Xenopus* cell line in which some oocyte-type 5S genes are translocated, some oocyte-type 5S genes replicate early and are expressed.**

In eukaryotes, chromosomal regions are replicated at different times in S phase (1, 2). Furthermore, particular regions appear to be replicated at the same time during successive S phases (3). In addition, there is evidence that tissue-specific genes replicate early in cells in which they are expressed. β-globin, which is expressed in the hematopoietic murine erythroleukemia cell line, replicates early in S phase in these cells (4), but it replicates late in S phase in HeLa cells, where it is not expressed (5). Calza *et al.* (6) have shown that the change in position during immunoglobulin gene rearrangement is accompanied by a change in time of replication for these genes. In this case, unexpressed immunoglobulin heavy-chain genes replicate late in two nonlymphoid cell lines, but they replicate early in two plasmacytoma cell lines in which they are expressed. Conversely, heterochromatic regions of chromosomes such as centromeres and telomeres, which are not typically expressed, are usually late replicating (7).

In *Xenopus laevis* there are two multigene families of 5S RNA genes (8): the oocyte-type 5S genes, which are expressed only in oocytes (9, 10), and the somatic-type 5S genes, which are expressed throughout development (10). There are 20,000 copies of the oocyte-type 5S genes and 400 copies of the somatic-type 5S genes per haploid genome (11), all of which are located at the distal ends of the long arms of most *Xenopus* chromosomes (12, 13). We found that, in somatic cells, the somatic-type genes replicate early in S phase, whereas the oocyte-type genes replicate late (14). These data show a correlation between early replication and expression for genes transcribed by RNA polymerase III that is consistent with the work noted above on genes transcribed by RNA polymerase II. The findings also provide support for the replication–expression model proposed by Gottesfeld and Bloomer (15) and by Wormington *et al.* (16). This model was formulated to explain the selective expression of somatic-type 5S RNA genes in somatic cells. At the time the pattern of 5S expression changes from the oocyte levels, there is a dramatic decrease in the level of the transcription factor TFIIIA, a protein required for transcription of both types of 5S genes (17). In oocytes, where both types of 5S genes are expressed, TFIIIA is present in large excess ($\approx 5 \times 10^5$ molecules per gene), whereas there is <1 molecule per 5S gene present in somatic cells (16, 18). However, factor depletion alone may not explain why only the somatic-type 5S genes are expressed. The replication–expression model suggests that genes that replicate early in S phase, in this case the somatic genes, have the first opportunity to bind TFIIIA stably and become active.

One prediction of the replication–expression model is that an alteration in the timing of replication of 5S genes should be accompanied by an alteration in their expression. We exploited a *Xenopus* somatic cell line in which some of the 5S genes are translocated to a pericentric position to investigate the relationship among chromosomal position, time of replication, and expression. Pardue *et al.* (12) had previously shown by *in situ* hybridization that, in addition to typical telomeric clustering of 5S genes, this line possesses a chromosome with two 5S clusters, one telomeric and the other pericentromeric. However, they did not determine which type of 5S gene was translocated to the pericentromeric location. Interestingly, Ford and Mathieson (19) showed by fingerprint analysis that there is some oocyte-type 5S RNA synthesized in this cell line. The cell line, originally established by K. A. Rafferty in 1968, has not been named. Based on the work reported here, we refer to it as TrXo (*Tr*anslocated *X*enopus *o*ocyte-type 5S genes). In the present study, we show that the translocated 5S RNA genes in TrXo are oocyte-type. In addition, the expression of some oocyte-type 5S genes in this cell line was confirmed by a new assay. Finally, we determined that in TrXo some oocyte-type 5S RNA genes in fact replicate early in S phase.

MATERIALS AND METHODS

Preparation of Biotin-Substituted Probes. pXlosp1, which contains a 312-base-pair insert of *X. laevis* oocyte-type 5S RNA spacer DNA (kindly provided by M. Wormington and D. D. Brown), was nick-translated in the presence of 16-C linker length biotinylated dUTP, using a nick-translation kit (Bethesda Research Laboratories) according to the manufacturer's instructions. Reactions were run for 90 min and unincorporated nucleotides were removed by Sephadex G-50 chromatography using the spin-column procedure (20). Probes were diluted to 4 μg/ml in 10 mM Tris·HCl, pH 7.6/1 mM EDTA/100 mM NaCl before use. pXls459, which contains a 459-base-pair insert of *X. laevis* somatic-type 5S RNA spacer DNA (13), was labeled in the same way.

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Abbreviations: BrdUrd, bromodeoxyuridine; FACS, fluorescence-activated cell sorter; HL, heavy light; LL, light light.

Chromosome Preparation for Electron Microscope *In Situ* Hybridization. Chromosome preparations were made from mitotically arrested *Xenopus* cultured cells (TrXo) obtained from M. L. Pardue. Exponentially growing cultures grown in 75% Dulbecco's minimal essential medium (DMEM; GIBCO) supplemented with 10% fetal calf serum (Biocell Laboratories) were arrested in metaphase by exposure to Nocodazole at 100 ng/ml (Sigma) for 18 hr. Metaphase cells were collected by shake-off, pelleted, and resuspended in 0.5 ml of DMEM with serum prior to cell lysis. Cells were lysed by adding the cell suspension to a droplet of 1% Nonidet P-40 (pH 8.5) on Parafilm to a final detergent concentration of 0.66%. After 6 min at 25°C, the droplet was pipetted gently but thoroughly to disperse the released chromosomes, and the suspension was centrifuged onto Parlodion carbon-coated gold electron microscope grids (Polyscience, Warrington, PA) through a cushion of 1 M sucrose (pH 8.5) for 15 min at 600 rpm in a GLC-2B centrifuge according to Hamkalo *et al.* (21).

Electron Microscope *In Situ* Hybridization. *In situ* hybridization was carried out as described by Narayanswami and Hamkalo (22) with minor modifications. After glutaraldehyde fixation and alkali denaturation for 10 min at 25°C, grids were hybridized for 12–15 hr at 30°C in 50% formamide (Bethesda Research Laboratories)/10% dextran sulfate (Pharmacia)/1 mM EDTA/10 mM Tris·HCl, pH 7.6/0.02% Ficoll-400/ bovine serum albumin (0.5 mg/ml) (nuclease-free; Bethesda Research Laboratories), *Escherichia coli* (40 μg/ml) DNA/600 mM NaCl/probe (800 ng/ml). After post-hybridization rinses, hybrid sites were located by sequential incubation of grids in a moist atmosphere in rabbit anti-biotin antibody (0.8 μg/ml), biotinylated goat anti-rabbit IgGs (1 μg/ml) (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline/NaCl, and streptavidin adsorbed to 20-nm colloidal gold particles (SAG20; Bethesda Research Laboratories), diluted 1:20 in 1% bovine serum albumin buffer (23). Unbound gold particles were removed by rinsing three times for 20 min each in 1% bovine serum albumin at 25°C with agitation, followed by a brief rinse in 0.4% Kodak Photoflo and air drying. Micrographs were taken on a JEOL 100C electron microscope operated at 80 kV.

Assay for Oocyte-Type 5S RNA in Somatic Cells. We developed a sensitive assay for oocyte-type 5S RNA to detect expression of oocyte-type 5S genes in TrXo. A 174-bp *Fnu*4HI/*Fnu*4HI fragment containing the *X. laevis* somatic-type 5S RNA gene was cleaved from the plasmid pXls11 (11) and subcloned into the *Hin*dIII site of the plasmid pSP64 (24) by use of synthetic *Hin*dIII linkers to generate the hybrid plasmid pSP64-Xls11. A 214-base ^{32}P-labeled RNA probe complementary to the *X. laevis* somatic-type 5S RNA was synthesized *in vitro* from *Bam*HI-linearized pSP64-Xls11 according to Melton *et al.* (24). The probe was then added to total RNA (200 ng) derived from one of the two cell lines or from *Xenopus* oocytes in 20 μl of 80% formamide/40 mM Pipes, pH 6.7/0.4 M NaCl/1 mM EDTA. After denaturation at 85°C for 10 min, the mix was incubated at 45°C for 4–5 hr. The hybrid was then recovered by ethanol precipitation and resuspended in 50 μl of 2× SET (1× SET = 150 mM NaCl/5 mM EDTA/50 mM Tris·HCl, pH 7.8) buffer for digestion with RNase A (10 μg/ml). Digestion was terminated by treatment with protease K and phenol extraction (24), and the products were analyzed by electrophoresis on an 8% polyacrylamide/8 M urea gel. RNase A digests the single-stranded region of the probe and cleaves at the mismatches in the hybrid to generate digestion patterns characteristic of oocyte and somatic 5S RNA.

Density Labeling, Cell Sorting, and DNA Isolation. Normal and TrXo *X. laevis* kidney tissue culture cells were grown to 30–50% confluency at 21.5°C and labeled with 50 μM bromodeoxyuridine (BrdUrd) in the presence of hypoxanthine (15 μg/ml) and amethopterin (15 μg/ml) for 3 hr prior to harvesting (14).

BrdUrd-labeled cells were trypsinized from monolayers stained with propidium iodide according to the method of Gray and Coffino (25). Populations of cells corresponding to different phases of the cell cycle were sorted on the basis of DNA content in the fluorescence-activated cell sorter (FACS). Cells were separated into G_1, four intervals of S phase, and G_2/M subpopulations by setting the appropriate windows with respect to fluorescence intensity after scanning the exponentially growing population.

Sorted populations of cells representing four segments of S phase, as well as G_1, G_2/M, and total S, were deproteinized and sheared according to Mariani and Schimke (26).

Newly replicated DNA [heavy light (HL)] was separated from unreplicated DNA [light light (LL)], by buoyant-density centrifugation in CsCl according to Guinta and Korn (14).

Gradient Transfer, Hybridization, and Quantitation. The fractions from each CsCl gradient were denatured, neutralized, and transferred to nitrocellulose by using a slot template according to Brown *et al.* (27). The filters were then hybridized with a ^{32}P-labeled nick-translated oocyte-specific pXlosp1 probe (13) in 50% formamide buffer according to Maniatis *et al.* (20). Densitometric tracings of the autoradiograms of these filters were made by using a Hoeffer densitometer and Hewlett Packard plotter and integrator. Since the length of the labeling time (3 hr) is long with respect to S phase (9 hr), cells collected in each stage of the cell cycle contain, in part, those cells whose labeling began in the previous stage. However, to estimate the amount of oocyte-type 5S DNA replicated in each fraction of S phase, we scanned the autoradiogram and determined the ratio of HL/(HL + LL). The ratio provides a relative measure of replication of the oocyte-type 5S genes in each portion of S phase. In each case, the ratio for G_1 phase was subtracted as background.

RESULTS

Translocation of the Oocyte-Type 5S RNA Genes. Electron microscope *in situ* hybridization was used to determine the origin of the translocated cluster of 5S genes. In these experiments, mitotic chromosomes from TrXo cells were hybridized with biotinylated pXlosp1 DNA, which is specific for oocyte-type 5S DNA (13), followed by immunogold detection of hybrids. Fig. 1 illustrates the hybridization patterns observed for prometaphase chromosomes (Fig. 1 *a* and *b*) and metaphase chromosomes (Fig. 1*c*). A pair of submetacentric chromosomes is shown in Fig. 1*a*, which is labeled at the telomeres of the long arms, the normal site of 5S RNA gene sequences (12, 13, 22), as well as at a second site juxtaposed to the centromere. Based on its size and arm ratio, the chromosome in Fig. 1*a* corresponds to that originally identified by Pardue *et al.* (12). A pair of subtelocentric chromosomes was observed with a similar labeling pattern (Fig. 1*b*), except that the atypical pericentric labeling was less intense and more centromeric. The subtelocentric chromosome represents another translocation, which may have occurred more recently. Alternatively, low centromeric labeling could have escaped detection after the short autoradiographic exposure used in the earlier study.

Fig. 1*c* shows both of these chromosomes in the same field. They were present in electron microscope preparations at similar frequencies and were seen in the majority of chromosome groups. Although the metaphase chromosomes in this figure are more compact and shorter than the prometaphase chromosomes shown in Fig. 1 *a* and *b*, the relative sizes and arm ratios are identical in both cases, indicating that these are the same chromosomes. When chromosomes from TrXo were hybridized with a probe (pXls459) specific for

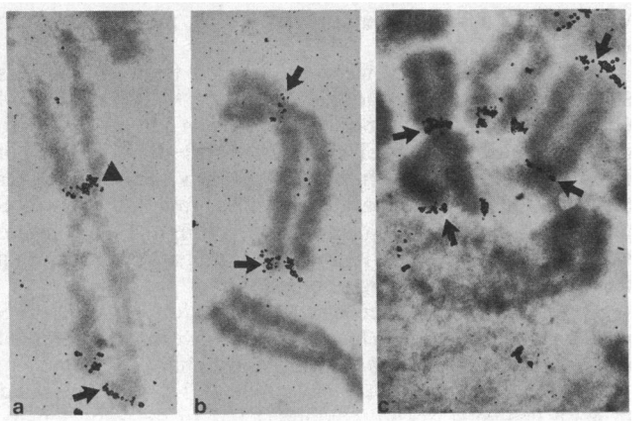

FIG. 1. Electron micrograph of *X. laevis* tissue culture cell mitotic chromosomes after *in situ* hybridization with biotinylated DNA containing the oocyte-type 5S DNA-specific sequence followed by immunogold detection with 20-nm colloidal gold particles. (×3000.) (*a*) A submetacentric prometaphase chromosome showing labeling at the telomeres of the long arms (arrow) and at a second site (arrowhead) close to the centromere. (*b*) A subtelocentric prometaphase chromosome showing a similar labeling pattern (arrows), but with less intense pericentric labeling. (*c*) Metaphase chromosomes from hybridized cells with both chromosomes visible. Hybrid sites are denoted by arrows.

somatic-type 5S DNA, only one pair of chromosomes was labeled and the label appeared at the telomere (data not shown). These data definitively identify the translocated genes as a subset of the oocyte-type 5S genes.

Translocated *X. Laevis* Cell Line Expresses Oocyte-Type 5S RNA. By RNase T1 and RNase A fingerprint analyses, Ford and Mathieson (19) demonstrated that TrXo, but not other *Xenopus* somatic cell lines, synthesizes a significant amount of oocyte-type 5S RNA. Several methods have been developed recently that distinguish more easily between oocyte- and somatic-type 5S RNAs, which differ at only 6 of 120 residues (28–30). We have used an RNA·RNA hybridization assay to confirm the original observation. The assay is diagrammed schematically in Fig. 2*A*; it is similar to the RNase mapping technique described by Melton *et al.* (24). One advantage of this assay over the others is that it did not require labeling or isolation of 5S RNA. Instead, the hybridization probe was labeled *in vitro* at high specific activity and thus provided higher sensitivity for detection of different types of 5S RNA. A labeled RNA probe complementary to the *X. laevis* somatic-type 5S RNA was synthesized by using the bacterial SP6 promoter. The probe was hybridized to total RNA derived from TrXo or another cell line or from oocytes. The hybrids formed between the probe and 5S RNA were subjected to RNase A digestion to eliminate the single-stranded regions of the probe and to cleave at cytosine and uracil mismatches in the hybrids.

As shown in Fig. 2*B*, the somatic-type 5S RNA from the normal cell line protected 120 bases of the probe (lane 2), representing a full-length gene, whereas the oocyte-type 5S RNA from oocytes produced a set of shorter fragments that were generated by RNase A cleavage at the mismatches (lane 3). Three predominant bands were observed under the conditions used; based on their sizes, the first two bands correspond to the two digestion products of ≈64 and ≈55 bases, respectively, generated by cleaving at either of the two consecutive mismatches occurring at base positions 55 and 56 of the oocyte-type 5S RNA. We have not identified the third band. A combination of oocyte-type and somatic-type digestion patterns was observed in RNA derived from TrXo (Fig. 2*B*, lane 1). Using densitometry, we determined that the bands characteristic of the oocyte-type 5S RNA represent ≈14% of the protected fragments in TrXo. This estimate

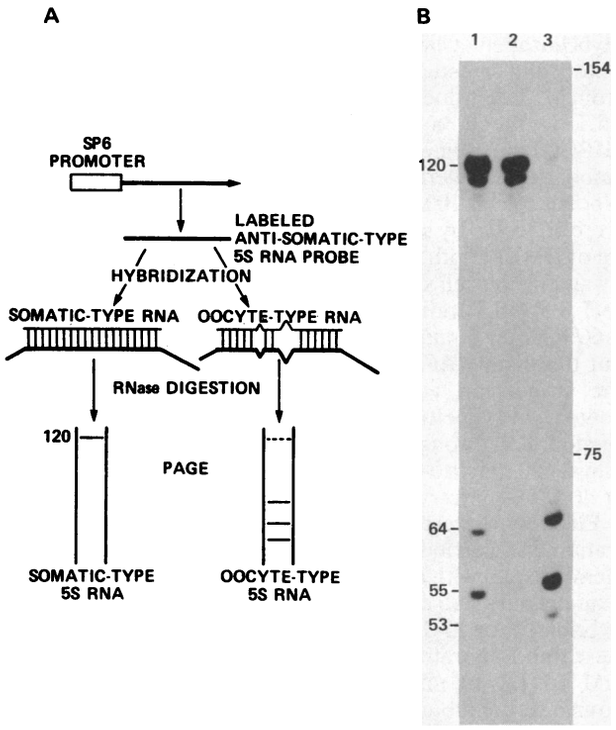

FIG. 2. (*A*) Hybridization assay for oocyte- and somatic-type 5S RNA in normal and TrXo *Xenopus* cell lines. (*B*) RNA-protected fragments from TrXo (lane 1), from the normal cell line (lane 2), and from oocytes (lane 3). The sizes of the fragments are indicated in bases.

agrees well with the 10–20% estimate for oocyte-type 5S expression determined by Ford and Mathieson (19). In contrast, no oocyte-type 5S RNA (<1%) could be detected in the normal cell line (Fig. 2*B*, lane 2).

Some Oocyte-Type 5S Genes Replicate Early in the Translocated Cell Line. Exponentially growing populations of *Xenopus* tissue culture cells were labeled for 3 hr (one-third of S phase) with BrdUrd, to increase the density of the newly replicated DNA. Cells were separated into four intervals of S phase by propidium iodide staining for DNA content, followed by sorting on the FACS. Cells that are more advanced in the cell cycle contain more DNA, bind more propidium iodide, and fluoresce more intensely. We achieved good separation of four populations of cells in different segments of S phase, designated S_1–S_4. Sheared DNA fragments from each segment were separated into newly replicated BrdUrd substituted DNA (HL) and unreplicated unsubstituted DNA (LL) by buoyant-density equilibrium sedimentation. Gradient fractions were transferred to nitrocellulose, hybridized with pXlosp1, and autoradiographed (14).

Fig. 3 shows that in TrXo, as in the normal cell line, the majority of the oocyte-type 5S genes replicate in S_3 and S_4. However, in contrast to the normal cell line, some oocyte-type genes do replicate in S_1 and S_2. Based on the densitometric analysis, we determined the ratios of replicating oocyte-type 5S genes to total oocyte-type 5S genes for each fraction of S phase and G_2. In TrXo the ratios for the four quarters of S phase and G_2 are 0.09, 0.12, 0.23, 0.24, and 0.28 for S_1–S_4 and G_2, respectively. In contrast, the values for the normal cell line are <0.01, 0.04, 0.13, 0.39, and 0.32. These values have been corrected for the background ratio in G_1 (TrXo, 0.03; normal cell line, 0.0) and are presented in histogram form in Fig. 4.

DISCUSSION

The mechanism by which *Xenopus* somatic cells selectively express somatic-type 5S genes and not the larger oocyte-type

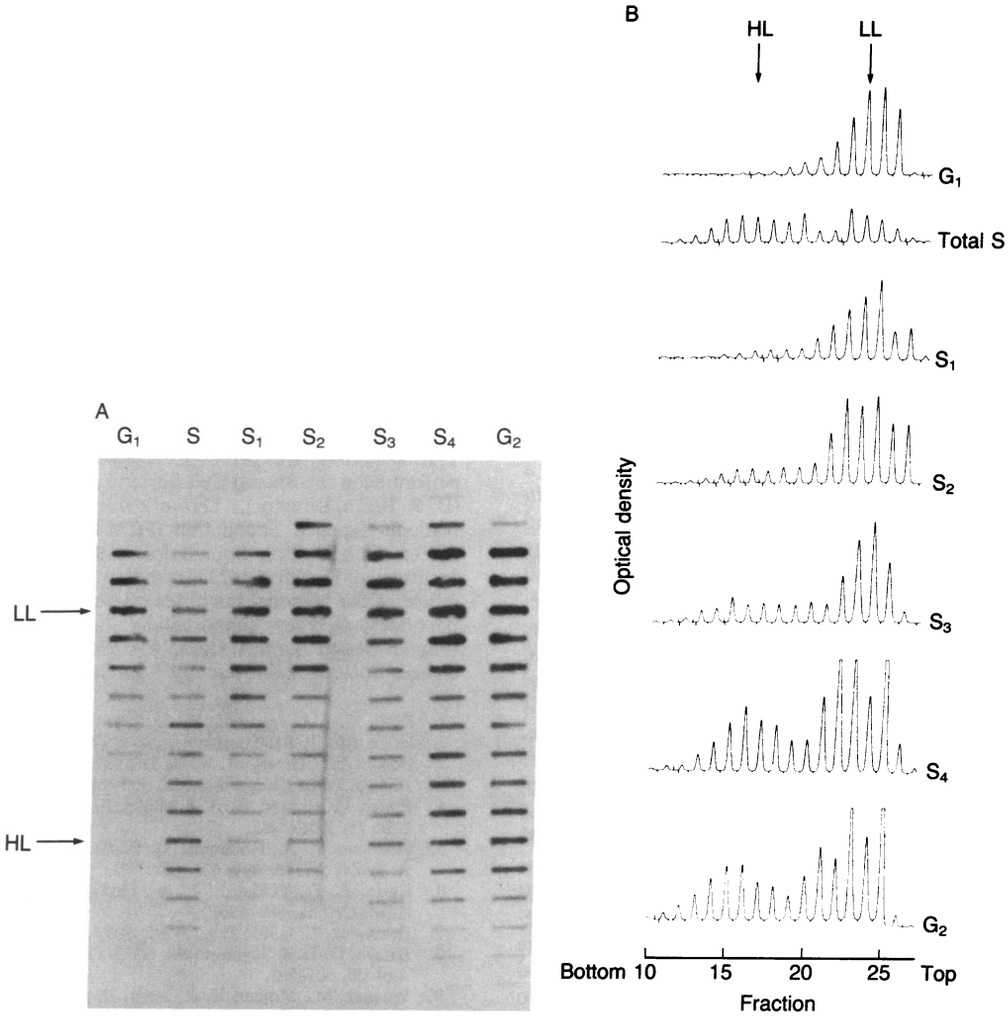

FIG. 3. Time of replication during S phase of the oocyte-type 5S genes in TrXo. (*A*) *Xenopus* cells were pulse-labeled with BrdUrd for 3 hr and separated into portions of the cell cycle on the FACS. DNA from cells in each portion of the cell cycle (G_1, S, and G_2) and from the four quarters of S phase (S_1, S_2, S_3, S_4) 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 gradient. Hybridization of a ^{32}P-labeled nick-translated probe specific for oocyte-type genes to slot blots of *Xenopus* DNA from each portion of the cell cycle is shown. LL shows the mean density of unreplicated oocyte (1.6920) 5S DNA; HL shows the density of replicated oocyte (1.7640) 5S DNA. (*B*) Autoradiogram in *A* was scanned by using a Hoeffer densitometer.

multigene family remains an unanswered problem in developmental biology. The replication–expression model (15, 16) proposes that the somatic-type 5S RNA genes replicate earlier in S phase than the oocyte-type 5S genes and, therefore, bind the small amount of TFIIIA present in somatic cells. Thus, the somatic-type 5S genes are expressed to the exclusion of the oocyte-type genes. This model is supported by the fact that TFIIIA forms stable active transcription complexes with 5S genes *in vitro* (31). Furthermore, in *Xenopus* somatic cells, the somatic-type 5S RNA genes replicate early in S phase, whereas the oocyte-type genes are late replicating (14).

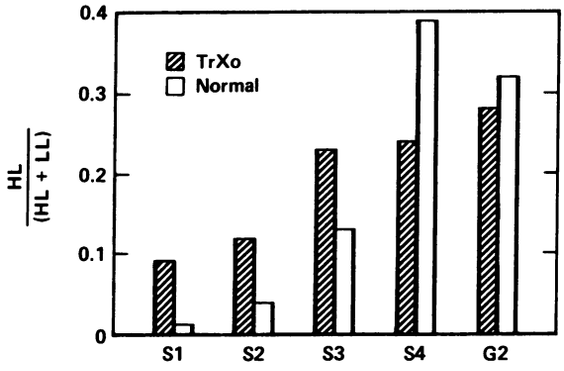

FIG. 4. Histogram comparing the relative ratios of replicating oocyte-type 5S DNA to total oocyte-type 5S DNA [HL/(HL + LL)] for each fraction of S phase and G_2 in the TrXo cell line and a normal *Xenopus* kidney cell line (14).

We have shown here that, in contrast to normal *Xenopus* cell lines in which <1% of the oocyte-type genes replicate in S_1, in TrXo ≈9% of the oocyte genes replicate in S_1. As in the normal cell line, the majority of oocyte-type genes continue to be late replicating. Using an independent assay, we have confirmed previous results showing that some of the oocyte-type 5S RNA genes are expressed in the TrXo cell line. We find that 14% of the 5S RNA in these cells is oocyte-type, in good agreement with the value of 10–20% reported by Ford and Mathieson (19). In addition to the expression of oocyte-type genes in TrXo, we find a change in the timing of replication of some oocyte-type genes. These observations support the replication–expression model.

Recently, Brown and Schlissel (31) demonstrated that

TFIIIA has a 25- to 200-fold higher affinity for the somatic-type than the oocyte-type 5S genes in injected embryos. They suggested that this could be sufficient to account for the >1000-fold differential expression of the somatic-type genes in somatic cells. However, in TrXo, some oocyte-type 5S RNA is synthesized despite the fact that the gene sequences are not altered (ref. 19; Fig. 2*B*); therefore, we conclude that selective expression of somatic-type 5S genes cannot be due solely to the inherent properties of the sequence. Additional control at the level of chromosomal position may be involved, as suggested by Ford and Mathieson (19).

The TrXo cell line has an unusual karyotype, which may be related to its altered 5S RNA expression. It was shown previously to contain, in one chromosome, 5S genes at both telomeric and pericentromeric regions (12). However, the identity of the translocated genes could not be determined because 5S complementary RNA, which cross-hybridizes to both types of genes, was used as a probe. We used a gene-type-specific probe (13) and electron microscope *in situ* hybridization to determine the identity of the translocated genes. In addition to the typical labeling at the tips of the long arms of *Xenopus* chromosomes, we observed labeling at secondary sites on two chromosomes with the oocyte-type-specific probe. Based on its relative size and arm ratio, one of these chromosomes is undoubtedly the same as that observed by Pardue *et al.* (12). The labeling intensity at the pericentromeric site of this submetacentric chromosome is comparable to that of the most heavily labeled telomeric site. Since there are 20,000 oocyte-type genes per haploid genome and 18 chromosome pairs, if these genes are equally distributed, there would be ≈1000 genes per telomeric locus. Based on this assumption, we estimate that there are at least 1000 oocyte-type genes translocated to this chromosome. However, the vast majority of oocyte-type 5S genes remain at their normal telomeric positions. Translocation of oocyte-type genes to a new site could in part be responsible for the expression of these genes as proposed by Ford and Mathieson (19). The other chromosome that exhibits two sites of labeling with the oocyte-specific probe is shorter and has a different arm ratio; labeling is directly over the centromere; and the centromeric labeling is considerably less intense than that on the submetacentric chromosome. The translocated genes on this chromosome may not have been detected in previous localization studies because of their low copy number or the fact that the translocation may have been a recent event.

Although we cannot directly prove that the translocated genes are those that are replicated early in S phase and are expressed in these cells, we find it unlikely that these are three independent random events. The simplest explanation for the properties of this cell line is that timing of replication, chromosomal position, and expression are causally related. Ford and Mathieson (19) proposed a causal relationship between the translocation and oocyte-type gene expression in this cell line. We suggest a hypothesis to extend this relationship to the timing of replication of the genes. The translocation of normally inactive genes to a site that is proximal to a region of "open" chromatin conformation may result in spreading of that "open" chromatin conformation into the translocated segment. This could then cause the genes to replicate early and be expressed. However, at the present time it is impossible to determine the cause and effect relationship between early replication and expression. This question could be addressed directly by introducing oocyte-type 5S genes, which are distinguishable from endogenous genes into new chromosomal locations in somatic cells. If some of these marked genes are early replicating, presumably by virtue of their proximity to an early replicating origin, it should be possible to determine whether these genes are also expressed.

There is abundant evidence that early replication and expression are correlated for genes transcribed by RNA polymerase II. It is possible that levels of RNA polymerase II transcription factors may fluctuate. Changes in concentration of specific transcription factors could lead to situations in which early replication of a gene would facilitate its expression by a mechanism analogous to that proposed for the 5S genes. Clearly, further studies in other differentially regulated systems will determine whether competition for transcription factors contributes to the correlation between early replication and expression in these systems.

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