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Oocyte and somatic 5S ribosomal RNA and 5S RNA encoding genes in *Xenopus tropicalis*

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ABSTRACT

We have investigated the structure of oocyte and somatic 5S ribosomal RNA and of 5S RNA encoding genes in *Xenopus tropicalis*. The sequences of the two 5S RNA families differ in four positions, but only one of these substitutions, a C to U transition in position 79 within the internal control region of the corresponding 5S RNA encoding genes, is a distinguishing characteristic of all *Xenopus* somatic and oocyte 5S RNAs characterized to date, including those from *Xenopus laevis* and *Xenopus borealis*. 5S RNA genes in *Xenopus tropicalis* are organized in clusters of multiple repeats of a 264 base pair unit; the structural and functional organization of the *Xenopus tropicalis* oocyte 5S gene is similar to the somatic but distinct from the oocyte 5S DNA in *Xenopus laevis* and *Xenopus borealis*. A comparative sequence analysis reveals the presence of a strictly conserved pentamer motif AAAGT in the 5'-flanking region of *Xenopus* 5S genes which we demonstrate in a separate communication to serve as a binding signal for an upstream stimulatory factor.

INTRODUCTION

The genes encoding 5S ribosomal RNA in *Xenopus* are developmentally regulated and have been the object of numerous structural and functional studies (for review, see ref.1). A major goal of the research using this system is to define the molecular basis for the differential expression of these structurally related transcription units. Two classes of 5S RNA genes have been defined in *Xenopus laevis* and *Xenopus borealis*; in *Xenopus laevis* the abundant oocyte 5S RNA genes consist of two structurally distinct families, the trace oocyte (1.300 copies per haploid genome) and the major oocyte genes (20.000 copies per haploid genome); in contrast, just 400 5S RNA genes constitute the somatic family (12,19,20). Both classes of 5S genes are transcriptionally active during oogenesis, but the

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oocyte genes are progressively inactivated during early embryogenesis, resulting in the selective expression of the somatic 5S genes in later stages of development (4,5,6).

Subspecies and species which constitute the genus *Xenopus* can be classified into two major phylogenetic groups on the basis of a number of biochemical and morphological criteria, such as immunological distance of albumin (2), chromosome number and DNA content (3), as well as the structural relation between adult globin genes (27). One of these two groups contains only a single species, the diploid *Xenopus tropicalis*, whereas the other group contains all other species, including *Xenopus laevis* and *Xenopus borealis*, which are tetra- or polyploid. *Xenopus tropicalis* is considered to resemble most closely the ancestor for the genes which existed prior to the separation into the *Xenopus tropicalis* and *Xenopus laevis* groups, about 100 millions of years ago.

The idea behind any comparative structural study is that conserved structure reflects conserved function. Thus, sequence information involved in and required for transcription regulation of the *Xenopus* 5S gene should be conserved amongst different species of the genus *Xenopus*. Moreover, sequences involved in the process which leads to the developmental profile of 5S gene expression in *Xenopus* should, if they exist, be distinguishing characteristics of oocyte and somatic 5S gene families.

We have investigated the 5S ribosomal RNA gene system in *Xenopus tropicalis*. The structure of oocyte and somatic 5S rRNAs and of 5S rRNA encoding genes was resolved. The sequences were subjected to a comparative functional and structural analysis with the corresponding elements in *Xenopus borealis* and *Xenopus laevis*.

MATERIALS AND METHODS

Structural RNA analysis

Total RNA from *Xenopus tropicalis* oocytes was isolated by phenol extraction of a total ovary homogenate low spin supernatant. Total RNA from liver tissue was obtained by phenol extraction of the total homogenate in extraction buffer (0.35 M Saccharose, 50 mM NaCl, 10 mM MgAc, 1.3 % Triton, 0.2 M Tris/Ac pH 8.5, 1 % SDS, 2 mM EDTA). 5S rRNA was isolated from these

crude, ethanol precipitated fractions by preparative gel electrophoresis.

Chemical and enzymatic sequence analysis was carried out on 5'- and 3'-end labelled RNA molecules according to standard procedures (22,23). Enzymatic probing of secondary/tertiary structure was performed as described previously (7).

Analysis of genomic DNA

Xenopus tropicalis genomic DNA was prepared from erythrocytes of single individuals according to standard procedures. DNA blots were probed with a ^{32}P-5'-end labelled fragment of *Xenopus laevis* 5S rRNA (nucleotides 41-120). Membranes were hybridized overnight at 68°C as described (24); following hybridization, filters were washed 3 times for 10 minutes in 2 x SSC at room temperature, dried and exposed.

Cloning of *Xenopus tropicalis* 5S rRNA genes

A genomic DNA library in Charon 24A (provided by G. Spohr, Geneva) was screened using the probe and hybridization conditions described above. The insert from a positive clone was subcloned into M13mp8 and subjected to DNA sequence analysis (25). Deletion mutants were obtained by use of the exonuclease III/mung bean nuclease approach (26).

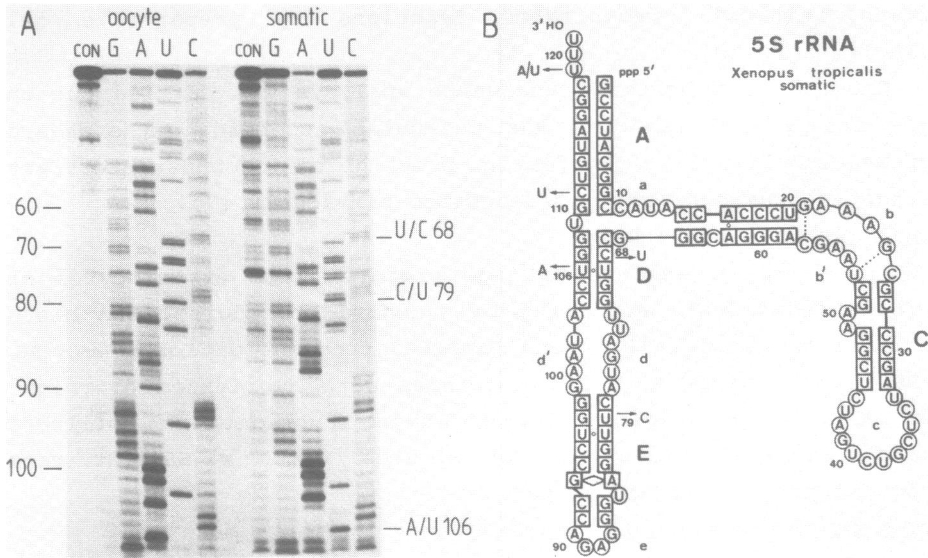
Analysis of transcription activity

In vitro transcription assays using a *Xenopus laevis* somatic 5S gene (pXls11; ref. 20) and a single *Xenopus tropicalis* oocyte 5S rRNA transcription unit in M13mp8 were performed in *Xenopus laevis* germinal vesicle extracts according to (21).

RESULTS

The only conserved difference between oocyte and somatic 5S rRNA in *Xenopus* is a C/U transition in position 79.

In order to investigate whether sequence heterogeneity as described for oocyte and somatic 5S rRNA in *Xenopus laevis* (4,6) exists in *Xenopus tropicalis*, we have isolated and sequenced 5S rRNA from oocytes and liver (Figure 1). Four sequence alterations could be detected; three of these, the C to U and U to C transitions, are structurally silent mutations (compensating base pair changes), since they maintain basepairing with the corresponding G residues. Conversely, the U to A transversion



<u>Figure 1</u>: Sequence analysis of oocyte and somatic 5S ribosomal RNA in *Xenopus tropicalis*.
5S ribosomal RNA was extracted from oocytes and liver (somatic) and subjected to chemical sequence analysis. The sequencing gel shown in (A) resolves the nucleotides homologous to the internal control region and reveals the sequence alterations indicated. The primary/secondary structure of *Xenopus tropicalis* somatic 5S rRNA is shown in (B); oocyte 5S rRNA specific sequence alterations are indicated.

at position 106 creates a new basepair in helix D in the oocyte 5S rRNA. A mismatch in this central position of helix D has been found to occur in another eucaryotic 5S RNA from *Rhizoctonia globularis* (10), suggesting that it does not significantly disrupt the secondary/tertiary structure in this portion of the molecule. The A16/A62 mismatch in helix B deviates from the general secondary structure of 5S RNA; however, mismatches in the same position occur in several other but not all of the *Xenopus laevis* and *borealis* oocyte 5S RNA molecules (10).

In a physical examination of the secondary/tertiary structure in *Xenopus tropicalis* oocyte and somatic 5S RNA by use of single- and double-strand specific nucleases as a probe (7) identical cleavage patterns were found with both RNA species, indicating that there are no significant conformational differences (data not shown). Similar conclusions were drawn

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             1o         2o         3o         4o         5o         6o
              '          '          '          '          '          '
Xto   GCCUACGGCC AUACCACCCU GAAAGCGCCC GAUCUCGUCU GAUCUCGGAA GCUAAGCAGG

Xlo   ---------- -C-------- -----U---U ---------- ------A--- --G-UA----
Xbo   ---------- -C---C---- -----U---- ---------- ---------- --G-U-----

Xts   ---------- ---------- ---------- ---------- ---------- ----------
Xls   ---------- -C-------- -----U---- ---------- ---------- --C-------
Xbs   ---------- ---------- -----U---- ---A------ ---------- --C-------

             7o         8o         9o        1oo        11o        12o
              '          '          '          '          '          '
Xto   GACGGGCUUG GUUAGUACCU GGAUGGGAGA CCGCCUGGGA AUACCAGGUG UUGUAGGCWU

Xlo   -U-----C-- ---------- ---------- ---------- ---------- -C------U-
Xbo   -C-----C-- ---------- ---------- ---------- ---------- -C------U-

Xts   -------C-- --------U- ---------- ---------- -----U---- C---------
Xls   -U-----C-- --------U- ---------- ---------- ---------- -C------U-
Xbs   -U-----C-- --------U- ---------- ---------- ---------- -C------U-
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Figure 2: Comparative sequence analysis of oocyte and somatic 5S ribosmal RNA in *Xenopus*.
Xenopus tropicalis (Xt), *Xenopus laevis* (Xl) and *Xenopus borealis* 5S rRNA sequences (as compiled in ref. 10) are aligned; only positions which deviate from the master sequence (Xto 5S rRNA) are indicated.

from a recent comparative structural analysis of oocyte and somatic 5S rRNAs from *Xenopus laevis* (8).

The number but not the position of oocyte 5S rRNA specific sequence alterations in *Xenopus tropicalis* (N=4) compares to the number of nucleotides changed in *Xenopus laevis* (N=6) and *Xenopus borealis* (N=6). We conclude that 5S gene expression in *Xenopus tropicalis* is developmentally regulated, similar to the situation described for *Xenopus laevis* and *Xenopus borealis*. The only conserved sequence alteration, which allows distinction between all oocyte and somatic 5S rRNAs in *Xenopus* is a C/U transition at position 79 (Figure 1).

Cloning and sequencing of a *Xenopus tropicalis* oocyte specific 5S RNA gene cluster

We have screened a *Xenopus tropicalis* genomic DNA library in Charon 24A (provided by Dr. G. Spohr, Geneva) using a *Xenopus laevis* oocyte 5S RNA fragment (nucleotides 41 to 120) as a probe. Several positive clones were isolated and the 1862 nucleotide insert in one of these subcloned into M13mp8 and subjected to DNA sequence analysis. It was found to contain six complete and two partial 5S RNA encoding elements (Figure 3). The length of the repeat unit is 264 nucleotides and the sequence is highly conserved; sequence heterogeneity was detected in only 6 posi-

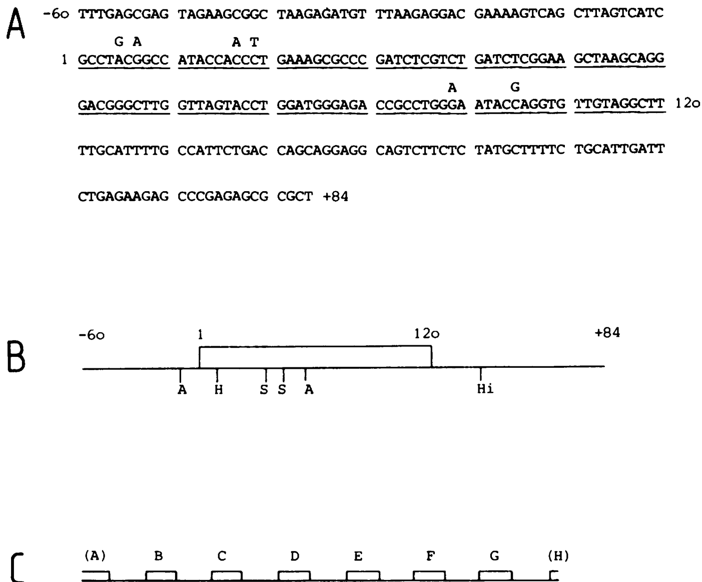


Figure 3: Structural description of an oocyte specific 5S RNA gene cluster in *Xenopus tropicalis*.
The sequence of the oocyte 5S rRNA encoding repeat unit in *Xenopus tropicalis* is shown in (A). Sequence alterations detected in other repeats of the 5S gene cluster described are indicated above the master sequence. Cleavage sites for restriction endonucleases used in the Southern blot experiment shown in Figure 4 are indicated in a schematic presentation in (B); the open bar represents the coding region of the 5S RNA gene, restriction endonucleases are: A - *Alu*I, H - *Hae*III, S - *Sau*IIIA, Hi - *Hin*fI. The arrangement of the six complete and two partial 5S RNA repeat units detected in the *Xenopus tropicalis* genomic DNA fragment analyzed is indicated in (C).

tions of the 5S RNA encoding portion, flanking sequences are absolutely conserved (Figure 3A). Similar microheterogeneities have been described for the oocyte 5S genes in *Xenopus laevis* (9). These observations illustrate the fact that sequence information based on RNA structure reflects predominant nucleotides in the corresponding positions of the 5S DNA, but the RNA derived sequence is not necessarily identical to any of the existing 5S RNA encoding genes. Since all of the *Xenopus tropicalis* 5S transcription units sequenced in our work contain the oocyte specific nucleotides of the coding region, we conclude that the cluster isolated is part of the oocyte 5S gene family.

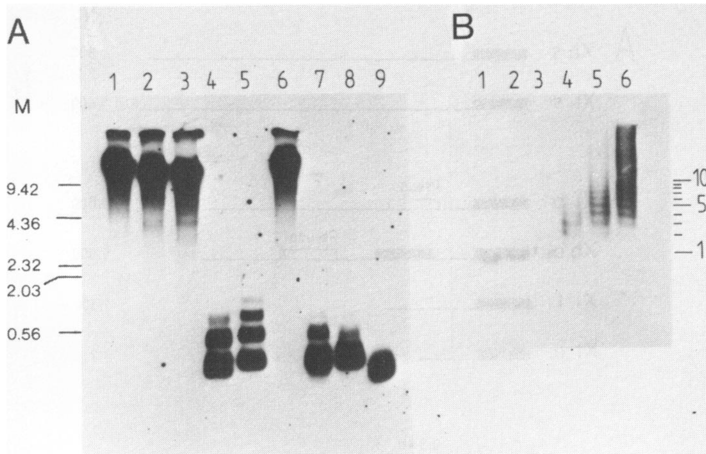
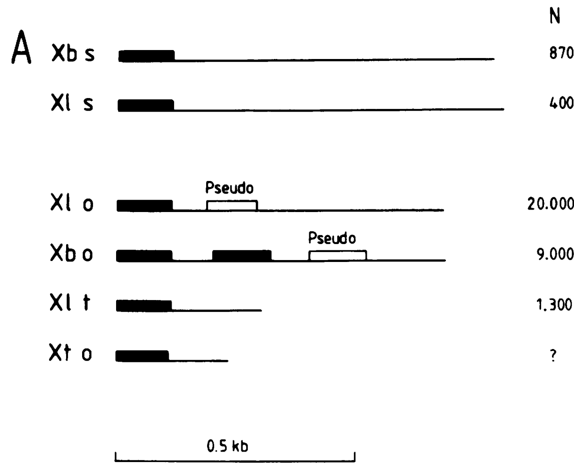


Figure 4: Genomic organization of 5S RNA encoding genes in *Xenopus tropicalis*.
Xenopus tropicalis genomic DNA was digested to completion and DNA fragments subjected to a standard Southern blot procedure using a *Xenopus laevis* oocyte 5S rRNA fragment (nucleotides 41-120) as a probe (A). Restriction endonucleases were: 1 - *Hin*dIII, 2 - *Hin*dII, 3 - *Bgl*II, 4 - *Alu*I, 5 - *Sau*IIIA, 6 - *Hpa*II, 7 - *Hae*III, 8 - *Hinf*I, 9 - *Dde*I.
In (B), *Xenopus tropicalis* genomic DNA was partially digested with decreasing amounts of *Alu*I (lane 1-6) and probed as in the experiment shown in (A). The number of 5S repeats visualized is indicated.

Results obtained in a genomic Southern blot experiment reflect the structural organization of the individual oocyte 5S genes which were isolated (Figure 4); the size of the DNA fragments detected corresponds to what one would predict from the restriction map shown in Figure 3B. The occurrence of multiple bands for some of the restriction enzymes utilized in this experiment indicates sequence micro-heterogeneity, as described above. The probe, nucleotides 41 to 120 of the *Xenopus laevis* oocyte 5S rRNA, and the hybridization conditions utilized, should also allow detection of somatic 5S RNA genes, since the number of mismatches with the probe is comparable for both gene families and even rare 5S genes should be visualized (Figure 2). It is therefore likely that the structural organization of somatic and of oocyte 5S gene families in *Xenopus tropicalis* is similar. We have also cloned and sequenced several 5S RNA



B

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          -4o        -3o        -2o        -1o
Xto    TAAGAGATGT TTAAGAGGAC GAAAAGTCAG CTTAGTCATC

Xbo    C--AGTC-TC A--GA--CGT C-------TT -ACTC-G--:
Xlo    CGCTGACAAG -C----A-C- -------GCC GC-GT-----
Xlt    CGCCCACA-G -CC-A-A-G- CC-----GCC GAGCT-----

Xbs    AG-AG-CA-C AC---G---G ---------- -C-T--GC--
Xls    AG-AG-CA-C AC-------G ---------- -C-T--GT--

Cons.  -R-------- -Y-RRRR--- --AAAGT--- -----T--TC
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Figure 5: Comparative structural analysis of oocyte and somatic 5S rRNA encoding transcription units.
The structure of oocyte (o) and somatic (s) 5S RNA genes in *Xenopus laevis* (Xl) *Xenopus borealis* (Xb) and *Xenopus tropicalis* (Xt) are outlined schematically in (A). Solid bars indicate coding regions, open bars pseudogenes; Xlt denotes the trace oocyte 5S RNA gene. Lengths, sequences and numbers of *Xenopus* 5S genes were taken from ref. 12,19,20, this communication.
Sequences found in the immediate 5'-flanking region of *Xenopus* 5S coding regions are compared in (B). Only nucleotides which differ from the master sequence (Xto 5S DNA) are indicated along with the deduced consensus sequence (CONS).

encoding 264 base pair fragments obtained from an *Alu*I digest of total genomic DNA from *Xenopus tropicalis*. However, these all exhibit the oocyte specific sequence characteristics, no somatic 5S RNA genes could be identified (data not shown). Thus, the structural organization of somatic 5S RNA genes and the presumed similarity to the oocyte 5S RNA genes in *Xenopus tropicalis* await final experimental proof.

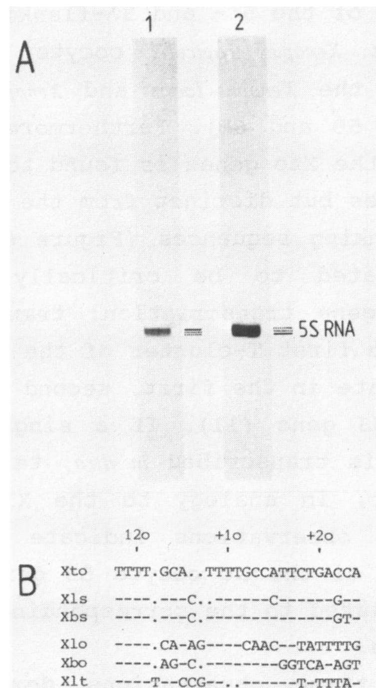

Figure 6: Comparative structural and functional analysis of termination signals for 5S gene transcription in *Xenopus*.
A single oocyte 5S RNA transcription unit from *Xenopus tropicalis* was transcribed *in vitro* by use of *Xenopus* germinal vesicle extracts; transcription products were separated on a sequencing gel (Panel A, lane 1) and compared to 5S RNA transcripts produced from a *Xenopus laevis* somatic 5S gene (pXls11; 20) under the same conditions (lane 2). Panel (B) shows a comparative sequence analysis of the 3'-flanking region of various *Xenopus* 5S genes; only nucleotides which differ from the master sequence (*Xenopus tropicalis* oocyte 5S DNA) are shown.

Comparative structural analysis of 5S RNA encoding transcription units in *Xenopus*

A schematic comparison of 5S gene structure in *Xenopus* suggests that the *Xenopus tropicalis* (Xt) oocyte 5S genes described in this communication differ significantly from the major oocyte-type 5S genes in *Xenopus laevis* (Xl) and *Xenopus borealis* (Xb) (Figure 5A). The Xt genes are devoid of pseudogenes and they lack AT-rich spacer regions, which are characteristics of the Xb and Xl oocyte 5S RNA transcription units. Moreover, a direct

sequence comparison of the 5'- and 3'-flanking regions in these genes reveals that *Xenopus tropicalis* oocyte 5S genes are most closely related to the *Xenopus laevis* and *Xenopus borealis* somatic 5S RNA genes (Figures 5B and 6B). Furthermore, the immediate 3' flanking region of the Xto genes is found to be very similar to the Xls and Xbs genes but distinct from the corresponding major Xlo and Xbo 3'-flanking sequences (Figure 6). The same region has been demonstrated to be critically involved in the termination of 5S gene transcription; transcripts produced *in vitro* terminate in the first T-cluster of the Xb somatic 5S gene, whereas they terminate in the first, second and third T-cluster of the Xl oocyte 5S gene (11). If a single *Xenopus tropicalis* 5S transcription unit is transcribed *in vitro*, termination occurs at the first T-cluster, in analogy to the Xl somatic 5S genes (Figure 6A). These observations indicate that transcription termination signals of the Xt oocyte 5S gene are structurally and functionally related to the corresponding Xb and Xl somatic 5S RNA gene elements.

In conclusion, these observations demonstrate that the structure of the *Xenopus tropicalis* oocyte 5S transcription unit differs significantly from the corresponding oocyte 5S RNA genes in *Xenopus laevis* and *Xenopus borealis*, but resembles the somatic type 5S RNA genes in these species.

DISCUSSION

Conserved sequence motifs in the immediate 5'-flanking region of *Xenopus* 5S RNA genes

A comparative sequence analysis performed on *Xenopus* 5S gene upstream sequences confirms the presence of conserved sequence elements reported by Korn and Brown (ref. 12); the strictly conserved pentamer motif AAAGT, located at position -18 to -14 relative to the start site of transcription, is preceded by a second, moderately conserved element, Y-RRRR at position -24 to -29 (Figure 5). Recently, we were able to demonstrate the functional significance of these elements; they serve as a binding site for a transcription stimulatory factor (Oei and Pieler, submitted). The occurrence of identical or related

sequence elements in the upstream region of other class III genes such as VAI RNA, tRNA, U6snRNA and other 5S RNA genes (as reviewed in ref. 12); Oei and Pieler, submitted) indicates a common function.

Interestingly, studies on the functional role of 5'-flanking sequences in transcription of the *Drosophila* 5S RNA gene (13) have revealed an upstream promoter element which overlaps with those sequence elements defined by Korn and Brown (ref. 12).

A C/U transition in position 79 of the internal control region in *Xenopus* 5S genes distinguishes somatic and oocyte gene families

A higher relative affinity of the 5S gene specific transcription factor IIIA for the somatic 5S genes has been implicated to play a role in the differential regulation of 5S gene expression in *Xenopus* (1). Sakonju and Brown (ref. 14) compared binding of TFIIIA to the *Xenopus laevis* trace oocyte gene and to the *Xenopus borealis* somatic 5S gene; they show that sequence differences which cluster in the 5'-portion of the internal control region (Figure 2) are responsible for a fourfold reduced affinity of the trace oocyte 5S genes for TFIIIA. If substitutions in position 53 and 55, supposed to be responsible for this effect, play a major role in the mechanism which leads to the developmental profile of 5S gene expression in *Xenopus*, they should be conserved in the *Xenopus tropicalis* system. However, inspection of the sequences reveals that *Xenopus tropicalis* oocyte and somatic 5S genes are identical in this critical portion of the internal control region (Figure 2). The only conserved substitution which distinguishes oocyte from somatic 5S RNA is a C to U transition in position 79. The same nucleotide, when mutated, was not found to have any detectable effect on transcription activity (15). Most recently, Bogenhagen and coworkers (ref. 16) reported that TFIIIA binds with an equal affinity to the major *Xenopus* oocyte and somatic type 5S genes. These observations indicate that since sequence changes which may result in an altered affinity for TFIIIA are not a conserved structural feature distinguishing the two differentially regulated 5S gene families, and since at least

some of the *Xenopus* oocyte and somatic 5S RNA genes have the same affinity for TFIIIA, differential affinity for the 5S gene specific transcription factor is not of primary functional significance with respect to the molecular mechanism which results in the developmental pattern of 5S gene expression observed.

Evolutinary considerations

The results presented on the structural organization of 5S RNA genes in *Xenopus tropicalis* suggest that the oocyte 5S RNA genes might be structurally and functionally related to the somatic 5S RNA genes, but they are distinct from the oocyte 5S RNA genes in *Xenopus laevis* and *Xenopus borealis*. The simplest interpretation of this apparently paradoxical observation is that somatic and oocyte 5S RNA genes in *Xenopus laevis* and *Xenopus borealis* have diverged rapidly as a consequence of a lack of selective pressure on the oocyte 5S RNA genes. The presence of pseudogenes (Figure 5) is in line with this idea. The close relatedness of oocyte and somatic 5S RNA genes in *Xenopus tropicalis* would then seem to reflect a more ancient situation with structural similarity of homologous, though differentially regulated genes coming from a common origin.

Due to the small number of sequence alterations between oocyte and somatic 5S rRNA from the various *Xenopus* species, construction of a phylogenetic tree which would allow us to trace back the differentiation of the two 5S gene families, as presented in other studies using the maximum parsimony method (17,18), was not carried out.

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