45S rDNA Repeats of Turtles and Crocodiles Harbor a Functional 5S rRNA Gene Specifically Expressed in Oocytes

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Abstract

In most eukaryotic genomes, tandemly repeated copies of 5S rRNA genes are clustered outside the nucleolus organizer region (NOR), which normally encodes three other major rRNAs: 18S, 5.8S, and 28S. Our analysis of turtle rDNA sequences has revealed a 5S rDNA insertion into the NOR intergenic spacer in antisense orientation. The insertion (hereafter called NOR-5S rRNA gene) has a length of 119 bp and coexists with the canonical 5S rDNA clusters outside the NOR. Despite the ~20% nucleotide difference between the two 5S gene sequences, their internal control regions for RNA polymerase III are similar. Using the turtle Trachemys scripta as a model species, we showed the NOR-5S rDNA specific expression in oocytes. This expression is concurrent with the NOR rDNA amplification during oocyte growth. We show that in vitellogenic oocytes, the NOR-5S rRNA prevails over the canonical 5S rRNA in the ribosomes, suggesting a role of modified ribosomes in oocyte-specific translation. The orders Testudines and Crocodilia seem to be the only taxa of vertebrates with such a peculiar rDNA organization. We speculate that the amplification of the 5S rRNA genes as a part of the NOR DNA during oogenesis provides a dosage balance between transcription of all the four ribosomal RNAs while producing a maternal pool of extra ribosomes. We further hypothesize that the NOR-5S rDNA insertion appeared in the Archelosauria clade during the Permian period and was lost later in the ancestors of Aves.

Key words: 5S rRNA genes, IGS, oocyte, rDNA amplification, specialized ribosomes, Archelosauria.

Introduction

The ribosomal RNAs (rRNAs) are essential structural and catalytic components of the ribosome, a cell protein-synthesizing machinery. The rRNAs are conserved across all forms of life from prokaryotes to eukaryotes. In most evolutionary groups, four types of rRNA molecules compose the ribosome. The 28S, 5.8S, and 5S rRNA are specific to the large ribosomal subunit (LSU, 60S), and 18S rRNA is a part of the small subunit (SSU, 40S). The complete 80S ribosome contains one molecule of each rRNA type. In the vast majority of multicellular animal organisms, the arrays of repeated genes for 18S, 5S rRNA and 28S rRNA are located in the nucleolus organizer region (NOR) on one or a few chromosome pairs (Olson 2011; Pederson 2011; Sochorová et al. 2018). In each rDNA repeat, these genes along with internal transcribed spacers (ITS1 and ITS2) and external transcribed spacers (5’ETS and 3’ETS) form a cluster that is transcribed by RNA polymerase I (Pol I) as a single transcription unit to synthesize a 45S pre-rRNA molecule. Transcription, processing of the 45S pre-rRNA into 18S, 5.8S, and 28S rRNAs, as well as assembling with ribosomal proteins into ribosomal subunits, occur in the nucleolus (Hernandez-Verdun et al. 2010; Pederson 2011; Henras et al. 2015). The clusters of rRNA genes in the NOR are separated by variable sequences of the intergenic spacers (IGS; Singer and Berg 1991). The copy number of rDNA repeats (rDNA cluster + IGS) can vary in the NOR from just a few to several hundreds. In most species, the molecular structure and organization of variable spacer sequences, in particular IGS, are poorly understood. The reason for this is the presence of extended internal GC-rich repeats that prevent IGS sequencing and contig assembly (Treangen and Salzberg 2011; Dyomin et al. 2017, 2019). The IGS sequences can contain important regulatory elements for the NOR rDNA activity (Agrawal and Ganley 2018; Abraham et al. 2020; Vydzhak et al. 2020).

The 5S rRNA genes have a small size of ~120 bp (Symonová 2019). In prokaryotes, yeasts, and some other lower eukaryotes, the 5S rRNA genes are incorporated into rDNA repeats as antisense sequence (Kramer et al. 1978; Bergeron and Drouin 2008; French et al. 2008). Some species with a similar localization of the 5S rRNA gene have also been described among Crustacea (Drouin et al. 2018). In each NOR, the 5S rRNA genes are separated by variable sequences of the intergenic spacers (IGS; Singer and Berg 1991). The copy number of rDNA repeats (rDNA cluster + IGS) can vary in the NOR from just a few to several hundreds. In most species, the molecular structure and organization of variable spacer sequences, in particular IGS, are poorly understood. The reason for this is the presence of extended internal GC-rich repeats that prevent IGS sequencing and contig assembly (Treangen and Salzberg 2011; Dyomin et al. 2017, 2019). The IGS sequences can contain important regulatory elements for the NOR rDNA activity (Agrawal and Ganley 2018; Abraham et al. 2020; Vydzhak et al. 2020).

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Nematoda (Vahidi et al. 1991; Wishart et al. 2002), some Mollusca (McElroy et al. 2021), and plants of the Asteraceae family (Garcia et al. 2017). Nevertheless, in the vast majority of eukaryotes, the SS rRNA genes are located in species-specific loci outside the 45S rDNA arrays (Singer and Berg 1991; Citigand and Williams 2011; Sochorová et al. 2018). Unlike 18S, 5.8S, and 28S rRNAs, which maturation and incorporation into the ribosomal subunits occur directly in the nucleolus (Henras et al. 2015; Klinge and Woolford 2019), a pathway of the SS rRNA incorporation into the ribosome is different and is more complex (Romanuik 2005; Smirnov et al. 2008). The 5S rRNA is exported to the cytoplasm, then returns to the nucleus and enters the nucleolus to be incorporated into the LSU (Smirnov et al. 2008). The reason why the 5S rRNA is synthesized independently of the other three rRNA types and passes along such a complicated route is largely unclear (Huang et al. 2020).

The ability to amplify the NOR rDNA to create extrachromosomal rDNA copies forming amplified nucleoli is a feature of oocytes in many animals with the so-called hypertranscriptional oogenesis (Dondua 2018). Among vertebrates, the phenomenon of ribosomal gene amplification during oogenesis is found in fish (Vincent et al. 1969; Raikova 1976; Thiry and Poncin 2005; Locati, Pagano, Ensink, et al. 2017; Locati, Pagano, Girard, et al. 2017), amphibians (Brown and Dawid 1968; Gall 1968; Macgregor 1968, 1972, 1982; Gall and Pardue 1969; Ficq and Brachet 1971; Gall et al. 2004), and some reptiles (Macgregor 1982; Guraya 1989; Calebaut et al. 1997; Uribe and Guillette 2000; Moore et al. 2008; Pérez-Bermúdez et al. 2012; Davidian et al. 2021). The strategy of amplified nucleoli provides early embryos with a sufficient number of ribosomes and thereby supports the protein synthesis until the zygotic transcription is activated (Davidson 1997; Uribe and Guillette 2000). The functional pool of ribosomes is necessary for proper formation of ribosomes. In amphibians and fish, the problem is solved by clustering oocyte-specific SS rRNA genes in separate loci outside the NOR (Pardue et al. 1973; Harper et al. 1983; Wormington et al. 1983; Locati, Pagano, Ensink, et al. 2017).

There are only a few data available on chromosomal localization and copy number of SS rRNA genes in reptile genomes (Sochorová et al. 2018; Cavalcante et al. 2020). The existing evidence of distinctive rDNA amplification strategy in turtle oogenesis (Macgregor 1982; Davidian et al. 2021) prompted us to study the molecular and evolutionary mechanisms underlying the phenomenon of rRNA gene amplification in oocytes of these animals in more detail.

Here, we present the first description of the NOR IGS structure in the turtles. Surprisingly, we found a copy of the SS rRNA gene inserted into the IGS sequences (hereafter called NOR-SS rRNA gene). We provide evidence of the evolutionary conservation of the NOR-SS rRNA gene in turtles and crocodiles and consider a possible evolutionary pathway of the NOR-SS rDNA in the Archelosauria clade. Using the red-eared slider Trachemys scripta as a model species, we demonstrate that the NOR-SS genes are expressed in oocytes only. Furthermore, the NOR-SS rRNA is incorporated into a functional pool of ribosomes. We propose and discuss a unique mechanism for balancing the ratio of different rRNA types in reptilian species with NOR rDNA amplification during oogenesis.

**Results**

**IGS Sequence in the Turtle rDNA Repeats**

In the NCBI database of turtle WGS, we found only one contig (NCBI MLDX1016458.1) containing two complete IGS sequences and three rRNA gene clusters (one complete and two fragments). It was referred to as the terrapin Malaclemys terrapin (Testudines, Emydidae). The structural elements of the terrapin rRNA gene cluster and their characteristics are shown in supplementary table S1, Supplementary Material online. The T. scripta rDNA repeat was assembled using the NCBI SRX217618 white-genome database obtained by Illumina technique and the above rDNA contig of M. terrapin as a reference. The resulting 15,058-bp long T. scripta sequence was found to be highly similar to the M. terrapin rDNA in both the rRNA gene cluster and IGS. The alignment of the T. scripta transcriptome reads (NCBI SRX121294) to the obtained rDNA sequence indicated the transcription start point of the 18S rRNA gene that corresponds to the beginning of 5ETS (supplementary fig. S1, Supplementary Material online). This region was found to be highly conserved between T. scripta and M. terrapin (supplementary fig. S2, Supplementary Material online). The IGS boundary with the 3’ETS appears to coincide with the very beginning of IGS internal repeats.

The definition of the IGS boundaries resulted in annotation of two IGS sequences of 6,780 and 6,734 bp long in contig MLDX1016458.1 (fig. 1). They contain four blocks (F, H, E, A) enriched with internal repeats of nine types, forming a complex regular structure (fig. 1, supplementary fig. S3 and data S1, Supplementary Material online). The H, E, A blocks and the 5’ETS sequence are separated by unique sequences of different lengths. Surprisingly, in the region located between repeat blocks E and A (fig. 1), we identified a sequence very similar to the 5S rRNA gene and directed opposite to the rRNA gene cluster. In T. scripta, a fragment of about 1,500–2,000 bp that partially overlaps repeat blocks H and E has been missing because the Illumina data did not allow the reconstruction of regions with long tandem repeats. It can be assumed that, similarly to terrapins, the IGS sequence size in T. scripta is about 6,000 bp. Importantly, in the red-eared slider, between repeat blocks E and A, we also found a sequence similar to the 5S rRNA gene (fig. 2). This finding was experimentally verified by PCR and Sanger sequencing. Furthermore, analysis of the WGS-contigs containing 18S and/or 28S rRNA genes in all available reptilian genome assemblies has revealed sequences similar to the 5S rRNA gene in the IGS of turtles and crocodiles (fig. 2). In what follows, we refer to the sequence inserted in the IGS as NOR-SS rDNA, as opposed to 5S rRNA genes clustered outside the NOR, which we refer to as canonical 5S rDNA.
The prevalent type of turtle IGS repeats is type A with the core motif CAGGTC revealed in all investigated contigs. Another most common repeat type is type H. They are both present in half of the turtle IGS studied (fig. 2, supplementary data S1, Supplementary Material online). We also found transposon and retrotransposon fragments in some IGS. The Gypsy-5_CPB long terminal repeat (LTR) retrotransposon was found upstream of NOR-5S rDNA in Chelonoidis abingdonii (fig. 2, supplementary data S2, Supplementary Material online). The Penelope-1_Crp non-LTR retrotransposon has been localized within the IGS in Alligator sinensis, CR1-2_Croc non-LTR retrotransposon, and DNA transposon hAT-8_Crp—in the IGS of A. mississippiensis (fig. 2, supplementary data S2, Supplementary Material online).

NOR-5S rDNA Structure and Prevalence in Vertebrates

In turtle genomes, only one conventional (canonical) 5S rDNA cluster was shown to exist outside the NOR (Cavalcante et al. 2020). Comparison between NOR-5S and canonical 5S consensus sequences in turtles and crocodiles provides strong evidence that NOR-5S is an inverted 5S rRNA gene inserted into the rDNA IGS in these reptiles (fig. 3A). Both the canonical 5S rRNA gene and NOR-5S rDNA sequences are of 119 bp.

We have also analyzed and compared sequences flanking both canonical 5S and NOR-5S rDNA upstream and downstream in a few turtle species (supplementary fig. S4, Supplementary Material online). The canonical 5S rRNA gene ends with GCT nucleotides. An oligo(T)-tails
downstream of the SS rRNA genes were shown to be typical transcription termination signals of Pol III (Korn and Brown 1978; Drouin and de Sa 1995). In turtles, between NOR-5S rDNA and oligo(T) sequences, there are two additional nucleotides (supplementary fig. S4, Supplementary Material online). This is consistent with the presence of additional nucleotides in unprocessed NOR-5S transcripts as described for certain Metazoan’s 5S rRNA (Vierna et al. 2013). The approximately 50-bp region upstream of the canonical 5S rRNA gene is rather conservative. It contains a WTAAA motif from -29 to -25 bp, which is likely important for transcription initiation in a number of vertebrates (Vierna et al. 2013). In the case of the NOR-5S rDNA, this motif is either absent or cannot be identified unambiguously (supplementary fig. S4, Supplementary Material online).

In contrast to Pol II, Pol III promoters are located within a transcribed sequence downstream of the transcription start site (Paule and White 2000). The SS and NOR-5S sequences in turtles have internal regions that resemble the internal control regions (ICRs) described for the *Xenopus laevis* 5S rRNA gene, where they are located between 48 and 100 bp downstream of the transcription start point (Pieler et al. 1987). This putative ICR contains three typical regions, characterized as parts of the Pol III promoter in *X. laevis*: the A box, an intermediate element (IE), and the C box (fig. 3B). Notably, the A box sequence in NOR-5S differs significantly from that in SS rDNA of *X. laevis* and canonical 5S rDNA of turtles. At the same time, each of the IE and C boxes in the NOR-5S has only one nucleotide substitution (fig. 3B). Box C seems to be the most important in the ICR of the SS rDNA sequence, as it recruits the transcription factor TFIIIA that attracts other factors and, finally, Pol III (Pieler et al. 1987; Hall 2005). Hence, we can assume that the NOR-5S gene has a functional Pol III promoter directing its transcription.

Variability of the NOR-5S rDNA sequence does not exceed 6% in turtles of different suborders (Pleurodira and Cryptodira), and 10% in alligators (*A. sinensis* and *A. mississippiensis*). The difference between the NOR-5S and the canonical 5S sequences in the same turtle species is 18–25%, and in alligators—18–22% (supplementary data S3, Supplementary Material online). As such, it was possible to identify the NOR-5S type without topological binding to the 18S and 28S rRNA genes. We collected all 5S rRNA gene sequences found in the available NCBI gene projects on Mammalia, Aves, Reptilia, Amphibia, and Actinopterygii to determine the NOR-5S rDNA insertion prevalence among vertebrates (supplementary fig. S5, Supplementary Material online). Sequences highly similar to the NOR-5S rDNA exist in all represented turtles (14 species) and crocodiles (3 species), but not in any of the representatives of Squamata (Reptilia) or vertebrates from the other analyzed classes (supplementary fig. S5, Supplementary Material online). The method of maximum likelihood was applied to the 5S rRNA gene sequences found in vertebrates, which separated the canonical 5S and the NOR-5S sequences into two evolutionary lines (fig. 4). Only turtles and crocodiles fall into both clusters due to the presence of both the canonical 5S and the NOR-5S in the same species (fig. 4). Importantly, the 5S rDNA tree topology does not reflect the true evolutionary relationships between taxa because of the short length of the analyzed sequence. It only records the main evolutionary variations in the 5S rDNA sequence.

**NOR-5S rRNA Gene Is Functional**

**NOR-5S rRNA Predicted Secondary Structure**

One of the ways to prove the functionality of the NOR-5S transcripts is to check whether this RNA folds into a typical secondary structure that can be part of the LSU. The ability of the sequence to adopt the correct secondary structure can be used to discriminate between genes and pseudogenes (Barciszewska et al. 2000). We predicted the secondary structure of both the canonical 5S and the NOR-5S rRNA sequences in turtles and crocodiles using the Andreescu et al. (2007) RNA energy model at +37 °C (fig. 5). The result is consistent with the eukaryotic 5S rRNA structure (Delilhas and Andersen 1982; Barciszewska et al. 1996, 2000).
According to Smirnov et al. (2008), Helix I has to stabilize the molecule and is potentially important for the incorporation into the LSU. Helix III and loop C seem to be also responsible for the 5S rRNA incorporation into the LSU due to the association with the ribosomal protein eL5. The loop E is responsible for TFIIIA binding. Helix IV and the terminal loop D are responsible for the interaction of the 5S rRNA with the 28S rRNA in the LSU. The majority of the 5S rRNA motifs completely coincide with the regions described by Vierna et al. (2013) as conserved in all metazoan 5S rRNAs. We find only a few nucleotide substitutions within loops B and D in the NOR-5S rRNA (shown in red in fig. 5). Despite these small variations, the NOR-5S rRNA secondary structure adopts a classical fold and appears to be appropriate for the incorporation into the ribosome.

**NOR-5S rRNA Is Specifically Transcribed Only in Oocytes**

In the available NCBI transcriptome databases, data on the Chinese pond turtle *Mauremys reevesii* transcriptomes from testis (NCBI SRX4393138), ovary (NCBI SRX4395162), and somatic tissues (NCBI SRX1469958) were found. Alignment of these transcripts to the canonical 5S and NOR-5S rDNA sequences using the Geneious software showed that the NOR-5S transcripts are located mainly in the ovary (fig. 6A).

We performed experimental validation of transcription of the canonical 5S and NOR-5S rRNA sequences using the Geneious software that showed the NOR-5S transcripts are located mainly in the ovary (fig. 6A). RT-PCR analysis revealed the canonical 5S transcripts in all tested samples, whereas NOR-5S transcripts were found only in the ovary (fig. 6B).
Growing oocytes within the ovary are surrounded by one or a few layers of follicular epithelium. In some reptilians and in birds, the cells of the follicular epithelium are known to participate in supplying the oocyte with various macromolecules including RNA (Schjeide et al. 1970; Taddei 1972; Rahil and Narbaitz 1973), which are the maternal stockpile for embryo early development (Davidson 1986). To find out the exact origin of NOR-5S in the T. scripta ovary, we performed the analysis of NOR-5S expression in the oocyte and follicular epithelium separately (fig. 6C). The primers used are shown in supplementary table S2 (nos. 1–2) and figure S6, Supplementary Material online. The NOR-5S mRNA was found only in the oocyte but not in follicular cells (fig. 6D). Thus, the amplified nucleoli (fig. 6C) must be the main source of NOR-5S transcripts in the oocyte. This suggestion was confirmed by RNA FISH with NOR-5S rDNA probe (see fig. 7).

In turtles, the rDNA amplification and formation of extrachromosomal nucleoli during oogenesis are well characterized (Callebaut et al. 1997; Davidian et al. 2021). Figure 7A shows the content of the oocyte nucleus (germinal vesicle, GV) manually dissected from T. scripta oocyte with a diameter of ~2 mm. It is worth noting that the GV content is very similar in oocytes with diameters of 0.5–5 mm, which are at the previtellogenic and early vitellogenic stages of oocyte growth. The GVs contain lampbrush chromosomes and large amounts of extrachromosomal RNA and DNA containing bodies (fig. 7A). Immunostaining for fibrillarin reveals multiple extrachromosomal nucleoli (fig. 7B, see also Davidian et al. 2021). RNA FISH to the oocyte frozen sections using NOR-5S rDNA probe (169 bp) showed fluorescence signals in the extrachromosomal nucleoli only (fig. 7C). According to Callebaut et al. (1997), in vitellogenic oocytes with a diameter of about 10 mm and more, the nucleoli move to the GV center.

**NOR-5S rRNA Incorporation into the Translationally Active Ribosomes in Oocytes**

To separate ribosomes and polysomes from lower-molecular weight components, the lysates prepared from T. scripta oocytes and liver were fractionated in a sucrose gradient. Six fractions from the gradient were manually collected relying on the polysome profile obtained by measuring the OD 260 in the case of the liver (supplementary fig. S7, Supplementary Material online). RNA from each fraction was extracted and cDNA was prepared as described in Materials and Methods. To assess the relative amount of different rRNAs in the fractions, we performed RT-qPCR with primers specific for canonical 5S, NOR-5S, and 28S rRNAs. The RT-qPCR data on ~2 mm previtellogenic oocytes revealed that canonical 5S rRNA is incorporated into ribosomes, although this 5S rRNA is present in the top of the gradient as well (fig. 8A). The NOR-5S rRNA is highly abundant only in the top (light) fraction (F1), which could be an indication of its storage in light RNP complexes. Previously, different studies have shown the storage of the oocyte-specific 5S rRNA in the form of 7S and 42S RNP particles in X. laevis previtellogenic oocytes (Denis and Mairy 1972; Picard and Wegnez 1979; Allison et al. 1991, 1995; Romaniuk 2005). The question arises whether the NOR-5S rRNA can be incorporated into the ribosomes at later stages of oocyte growth. Therefore, we extended our studies to oocyte growth in successive stages. In ~5 mm previtellogenic oocytes, we observed a similar abundance pattern of both the NOR-5S rRNA and canonical 5S rRNA through the fractions. In the top fraction (F1), both types of 5S rRNAs are highly enriched, suggesting the presence of both rRNAs in a free form or their storage in light RNP complexes. In the 80S fraction (F3) and the polysome fractions (F4–F6), we see not only the canonical 5S but also the NOR-5S rRNA molecules (fig. 8B). We assume that such a distribution of NOR-5S rRNA by fractions may prove to be the inclusion of a certain amount of NOR-5S rRNA into the ribosomes.

We then tested ~10 mm yolk oocytes, and here, we found a striking difference in the pattern of the two types of 5S rRNA (fig. 8C). The canonical 5S rRNA was observed mostly in the light fractions (F1, F2), whereas the NOR-5S rRNA was less in these fractions, in contrast to what was observed before in the early-stage oocytes (~2 and ~5 mm). In vitellogenic oocytes, the NOR-5S rRNA was highly enriched in both the 80S fraction (F3) and the polysome fractions (F4–F6). The dynamics of accumulation of each of the rRNA types in different fractions through the three stages of oocyte

**Fig. 5.** The Trachemys scripta canonical 5S and NOR-5S rRNA secondary structures predicted using the Andronescu et al. (2007) RNA energy model at +37 °C. The nucleotides that are highly conserved in the 5S rRNA among all the metazoans studied (Vierna et al. 2013) are marked in black. The nucleotides differing from the conservative ones are marked in red.

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To our knowledge, the 5S rDNA insertion (NOR-5S rRNA gene) into the IGS sequence found in this study is the first known example of such a peculiar NOR organization in the representatives of Vertebrata. Importantly, we obtained evidence of functional activity of the NOR-5S rDNA in turtles. Firstly, the sequence of the NOR-5S gene is similar to the canonical 5S gene, and accordingly their predicted secondary structures are also similar. Secondly, the NOR-5S rDNA has all the necessary regulatory motifs to initiate transcription of the 5S rDNA. Thirdly, we detected transcripts of the NOR-5S gene in growing oocytes experimentally. And finally, we demonstrated incorporation of the NOR-5S rRNA into the ribosomes of vitellogenic oocytes.

**Discussion**

**Structural and Functional Aspects of the NOR-5S rDNA Insertion**

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Most eukaryotes are known to have only one cluster of the canonical 5S rDNA per chromosome set (Sochorová et al. 2018). In *Podocnemis expansa* and *P. unifilis* turtles, the only cluster of 5S rRNA genes outside NOR was found to be localized on chromosome 13 (Cavalcante et al. 2020). As for the NOR, the turtles of various families were shown to possess a single NOR pair per diploid chromosome set (Bickham and Rogers 1985; Cleiton and Giuliano-Caetano 2008). In the *T. scripta* karyotype, a single NOR was confirmed to be located on chromosome 14 using fluorescence in situ hybridization (Cleiton and Giuliano-Caetano 2008). Among reptiles, the phenomenon of NOR DNA amplification and formation of extrachromosomal nucleoli during oogenesis has been described for turtles and crocodiles (Macgregor 1982; Guraya 1989; Callebaut et al. 1997; Uribe and Guillette 2000; Pérez-Bermúdez et al. 2012; Davidian et al. 2021). However, the mechanism of balancing coexpression of all four rRNAs remained unknown. Our finding of functionally active NOR-5S rDNA suggests that its insertion into the NOR might help to balance expression of all four ribosomal genes. During oogenesis of studied species, the 5S genes are amplified as part of the entire NOR DNA and thus participate in the formation of numerous extrachromosomal nucleoli generating the maternal pool of extra ribosomes.

In vitellogenic oocytes, the NOR-5S rRNA, by contrast to the canonical 5S rRNA, is mainly deposited in the ribosomal and polysomal fractions. It is evident that the two types of 5S rRNA genes in turtles (and likely crocodiles) are expressed differentially, just as in the case of the fish and amphibian genomes (Picard and Wegnez 1979; Davidson et al. 2012; Davidian et al. 2021).

![Fig. 7. Amplified nucleoli in *Trachemys scripta* oocytes. (A) Nucleus isolated from an oocyte at previtellogenic stage. LBC and multiple extrachromosomal bodies, among them the amplified nucleoli. Sytox Green fluorescence. (B) Cryosection through the juvenile *T. scripta* ovary. Immunohistochemistry with antibody to fibrillarin (a marker of the nucleolus), green fluorescence, counterstained with DAPI (gray). (C) RNA FISH to the ovary frozen section using NOR-5S rDNA probe (169 bp). FC, follicular cells; LBC, lampbrush chromosomes; N, nucleus.](image1)

![Fig. 8. RT-qPCR-based visualization of polysome profiles from the *Trachemys scripta* oocytes of different growth stages. The result of RT-qPCR data analysis for previtellogenic oocytes (A, B), vitellogenic oocytes (C). The columns marked in black correspond to 28S rRNA, in gray—to canonical 5S rRNA, in red—to NOR-5S rRNA. Y-axis shows normalized ribosomal RNA quantity, X-axis represents sucrose fractions (F) from 1 (top) to 6 (bottom). Error bars display the standard deviation of RT-qPCR technical replicates.](image2)
groups of dinosaurs and pterosaurs. This could happen in the second half of the Permian period (Shen et al. 2011; Chiari et al. 2012; Luo et al. 2019). The 5S rRNA gene sequence later disappeared from the ribosomal repeats in the avian evolutionary lineage. Noteworthy, along with the absence of the 5S rDNA insertion into the NOR IGS, the representatives of modern Lepidosauria (in particular, Squamata) and Aves lack the process of rDNA amplification and extrachromosomal nucleoli formation during oogenesis (Gaginskaya et al. 2009; Koshel et al. 2016). However, we cannot make any assumptions about either ribosomal gene amplification or the NOR-5S insertion existence in the oocytes of dinosaurs and the ancestors of birds, yet.

At the moment, there are no serious prerequisites for convincing conclusions about the mechanism of the appearance of the 5S rRNA gene in the ribosomal repeat sequence. But after embedding in the IGS, the 5S sequence apparently evolved in accordance with the laws common to tandem repeats. The identity of two NOR-5S rDNA sequences within contig MDX1016458.1 suggests that these sequences are really evolving by the mechanisms responsible for the concerted evolution of tandemly repeated multigene families (see Drouin and de Sá 1995).

The occurrence of the 5S rRNA gene insertion within the ribosomal repeat in taxa characterized by the formation of extrachromosomal nucleoli in oocytes through rDNA amplification could be a molecular aromorphosis that significantly changed the strategy of ribosome synthesis during oogenesis. In this case, the problem of an imbalance between the number of 18S, 5.8S, 28S rRNA genes, on the one hand, and the number of 5S rRNA genes, on the other, is solved elegantly. We do not rule out the possibility that the large changes of the 5S rDNA sequence insertion could be a consequence of the manifestation of new, as yet unknown, functions of the NOR-5S rRNA in reptilian oogenesis.

**Materials and Methods**

**Biological Materials and Ethics Statement**

Fresh *T. scripta* tissues of the ovaries, testes, heart, liver, skeletal muscles obtained from mature and immature females were flash-frozen in liquid nitrogen and stored at −80 °C. The procedures related to manipulation of animals were approved by the Ethical Committee of Saint Petersburg State University (Statement no. 131-03-3 issued on June 1, 2017) in accordance with the National Research Council (2011).

**Bioinformatic Analyses**

The NCBI database of turtle WGS (https://www.ncbi.nlm.nih.gov/genome/?term=Testudines, last accessed November 13, 2021) was searched for genome contigs containing IGS sequences flanked with 28S and 18S rRNA genes. For identifying the 28S and 18S rRNA genes, we used the corresponding evolutionary conserved sequences from the chicken rRNA gene cluster (Dyomin et al. 2016). The search for nucleotide sequences containing fragments of rRNA genes and IGS sequences was performed using the NCBI BLAST algorithm. Reference assembly, nucleotide composition, and coverage assessment, dot-plot analysis, sequence annotation, and 5S
rRNA secondary structures modeling were performed using Geneious 9.1 (http://www.geneious.com/, last accessed November 13, 2021). Assembly of the terrapin turtle *M. terrapin* and red-eared slider *T. scripta* rRNA repeats was performed using the Geneious algorithm. The SS rRNA secondary structures were predicted using the Andronescu *et al.* (2007) RNA energy model at +37 °C implemented in Geneious 9.1. Repbase on an online basis (http://www.girinst.org/rebase/index.html, last accessed November 13, 2021) was used for annotated repeats search. Primers were designed using Primer3 software. Alignment of nucleotide sequences using the Clustal W method and molecular phylogenetic analysis using the maximum likelihood inference algorithm were performed in MEGA 7.0 (http://www.megasoftware.net/, last accessed November 13, 2021) to generate a p-distance matrix. The Kimura 2-parameter model with Gamma Distributed was chosen as the optimal evolutionary model (supplementary data S4, Supplementary Material online). The reliability of the tree topology was estimated using the Bootstrap-test (3,000 replications).

To determine whether the NOR-5S rRNA is transcribed in different turtle tissues, we analyzed raw transcriptomic data available in NCBI (NCBI BioProjects: PRJNA481226; PRJNJA305199; PRJNA82237). We used *M. reevesii* transcripts of the testis (NCBI SRA: SRX4393138), ovary (NCBI SRA: SRX4395162), and somatic tissue (liver, muscle, and spleen) (NCBI SRA: SRX1469958). Alignment of the transcripts to the canonical SS and NOR-5S rDNA sequences of *M. reevesii* was performed using the Geneious algorithm.

**RNA Isolation and Gene-Specific RT-PCR Analysis**

Traditional the RNA purification method using TRizol reagent (GibcoBRL, USA) was performed for tissue samples according to Rio *et al.* (2010). Removal of genomic DNA from RNA samples was performed by DNase I (Thermo Fisher Scientific, USA) for 30 min at +37 °C as suggested by the manufacturer. RNA was purified and concentrated by phenol/chloroform/isoamyl alcohol extraction followed by subsequent ethanol precipitation. For cDNA preparation, the SuperScript III (Thermo Fisher Scientific, USA) Reverse Transcription kit was used as suggested by the manufacturer. For transcription analysis, RT-PCR was carried out by using Phusion High-Fidelity PCR Kit (New England Biolabs, USA) with corresponding F and R primers (see supplementary table S2, nos. 1–4, and fig. S6, Supplementary Material online). The RT-PCR program: denaturation for 1 min at 98 °C, 35 amplification cycles, each consisting of denaturation at 98 °C for 10 s, annealing at 63 °C for 15 s, primer extension at 72 °C for 10 s, and final elongation at 72 °C for 5 min. As a negative control, we used the same amount of RNA treated with DNase I not reverse-transcribed.

**Separation of Oocyte and Follicular Epithelium**

To separate the oocyte from follicular epithelium, we first collected follicles in PBS with 5 mM CaCl₂ and 1 mg/ml collagenase (no. c6885, Sigma). The incubation with collagenase was carried out by shaking at room temperature for about 1 h. Then the follicles were washed with PBS, and the oocytes were manually separated from the rest of the follicular epithelium using Leica MZ12 stereomicroscope and preparative needles. The oocytes and their follicular envelopes were collected in different tubes on ice, flash-frozen in liquid nitrogen, and stored at −80 °C. Oocytes of ∼2, ∼5, and ∼10 mm diameters were used.

**Lysate Preparation and Centrifugation in Sucrose Gradient**

The oocytes were homogenized with 20 strokes using Eppendorf pestle in the lysis buffer (20 mM Tris HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% Triton X-100), supplemented with 0.1 mg/ml cycloheximide and complete EDTA-free protease inhibitors (Roche). For the oocytes of ∼2 and ∼5 mm, the volume of the lysis buffer was 400 μl, for the oocytes of ∼10 mm, the volume was 1 ml. In parallel, the *T. scripta* liver lysate was used as a centrifugation quality control. The liver was mechanically disrupted in 1 ml of lysis buffer with the Dounce homogenizer (20 strokes with pestle B). To remove cellular debris, the homogenates were centrifuged at 12,000 × g for 10 min at +4 °C. After that, the lysates were loaded on a 10–50% linear sucrose gradient in SW41 tubes (Beckman Coulter, USA). Gradients were made in buffer (20 mM Tris HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT). After centrifugation at 35,000 rpm for 3 h at +4 °C, the gradients were analyzed by measuring absorbance at 260 nm and the selected fractions were collected manually. The gradient from liver lysate served as a reference to determine the position of monosome (80S) and polysome fractions. Further analysis using RT-qPCR was performed on six pooled fractions. The scheme of gradient fractionation is shown in supplementary figure S57, Supplementary Material online.

**RNA Isolation from Sucrose Fractions and RT-qPCR**

Before RNA extraction, 0.75 ng of in vitro synthesized mRNA of firefly luciferase (FLuc) was added to 300 μl of each of six pooled fractions for normalization. RNA extraction was performed using TRizol LS (no. 10296028, Invitrogen) and precipitated with glycogen and isopropanol according to the manufacturer’s recommendations followed by DNase I (Thermo Fisher Scientific, USA) treatment in 20 μl for 30 min at +37 °C. RNA was then purified using phenol-chloroform extraction and ethanol precipitation. The RNA pellet was resuspended in 12 μl nuclelease-free water. Four microliters of RNA from each fraction were reverse-transcribed using SuperScript III (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. cDNA was diluted 10-fold and 1 μl was used in a 10 μl reaction for SYBR green detection on a CFX96 machine (Bio-Rad, USA). RT-qPCR was performed in duplicates using Phusion High-Fidelity PCR Kit with 1X Phusion GC Buffer, 0.2 mM dNTP, 1X SYBR Green (no. S7653, Invitrogen), 0.5 μM corresponding F and R primers (see supplementary table S2 and nos. 3–6, Supplementary Material online). The amplification protocol was 98 °C for 1 min, 30 cycles of 98 °C for 10 s, 63 °C for 15 s, 72 °C for 10 s; followed by melting curve determination. No-template controls were also included. Ct values for different
rRNAs were normalized on FLuc Ct values to reduce variations between fractions due to the RNA isolation and cDNA preparation steps.

Cytological Material
Turtle oocyte nuclei were manually dissected from the growing oocytes of \( \sim 2 \) mm diameter and stained with Sytox Green (Molecular Probes, USA) accordingly with Saifitdinova et al. (2017). Notably, the GV and GV content manual dissection is possible from the previtellogenic oocytes only, not the vitellogenic ones (Davidian et al. 2021). In vitellogenic oocytes, the GV is getting smaller and tightly pressed against the oocyte shell. Cytosections were prepared, immunostained, and in situ hybridized just as described by Davidian et al. (2021). The NOR-5S probe (169 bp) was amplified and labeled with digoxigenin by PCR using developed F and R primers (see supplementary table S2, no. 2, and fig. S6, Supplementary Material online). The preparations were examined using Leica DM4000B epifluorescence microscope and Leica TCS SP5 confocal laser scanning microscope. The final images were produced using the maximum intensity projection function in Fiji software.

Supplementary Material
Supplementary data are available at Molecular Biology and Evolution online.

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Data Availability
Malaclemys terrapin nucleotide sequence data reported are available in the Third Party Annotation Section of the DDBJ/ENA/GenBank databases under the accession number TPA: BK059377.

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