

ORIGINAL PAPER

AMPHIBIANS AS MODEL ORGANISMS FOR STUDYING THE DYNAMICS OF EUKARYOTE GENETIC MATERIAL ARCHITECTURE

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Keywords

amphibians,
chromatin remodelling,
gametogenesis,
DNA methylation,
lampbrush
chromosomes,
ribosomal gene
amplification.

Abstract

Amphibians have played a key role in the elucidation of the mechanisms of early development over the last century. Much of our knowledge about the mechanisms of vertebrate early development comes from studies using *Xenopus laevis*. *Xenopus* sp. is a major contributor to our understanding of cell biological and biochemical processes, including: (1) chromosome replication; (2) chromatin, cytoskeleton and nuclear assembly; (3) cell cycle progression and (4) intracellular signaling. Amphibian embryos remained the embryos of choice for experimental embryology for many decades. European embryologists used predominantly urodele embryos (such as *Triturus*) and embryos of the frog *Rana temporaria*, which is related to the North American species *Rana pipiens*.

Using light, fluorescence, transmission electron microscopy (TEM) and molecular investigations, some peculiar aspects of chromatin and chromosome organization and evolution in oogenesis and spermatogenesis of amphibians were investigated. We have focused our investigations on dynamics of the chromatin structure in different stages of development.

Introduction

The germinal cells show a particular architecture of genetic material, with functional implications because of their very important role in heredity.

The task of oogenesis is to construct a large cell containing a large and complex dowry of resources for construction of the embryo before it can produce them itself or obtain them from its environment. One distinction of amphibian oogenesis is the formation of lampbrush chromosome during the major phase of oocyte growth and differentiation [1].

Lampbrush chromosomes are quite peculiar. They are large and they look quite different from any other form of chromosome and they have offered a successful medium through which it has been possible to draw valid conclusions at the molecular level on the basis of observations and experiments using light microscopy. Lampbrush chromosomes have proved uniquely valuable in two respects. First, they are transitory structures that exist during an extended diplotene of first meiotic division. The chromosomes go from a compact telophase form at the end of the last oogonial mitosis, become lampbrushy and then contract again to form perfectly normal first meiotic metaphase bivalents. Secondly, their most conspicuous feature is widespread RNA transcription on hundreds, and in some cases thousands, of loops that project laterally from the main axis of the

chromosome at short intervals along its length. Therefore, it has been possible to exploit lampbrush chromosomes in the study of chromosome organization and gene expression during meiotic prophase [1], and in studies on the molecular and supramolecular morphology of RNA transcription [1]. The name lampbrush comes from Rückert, who likened the object to a 19th Century lampbrush, equivalent to the 20th Century test-tube brush [1].

Lampbrush chromosomes are characteristic of growing oocytes in the ovaries of almost animals. The chromosomes are greatly elongated diplotene bivalents, sometimes reaching lengths of a millimeter or more [2].

The early diplotene nucleus is also characterized by the formation of large numbers of nucleoli (up to 1500 in *Xenopus*), which soon become distributed beneath the nuclear membrane [2]. These nucleoli are the morphological expression of a phenomenon known as a *specific gene amplification* [3]. Gene amplification is an adaptative response for meeting the synthetic requirements of the egg, in this case the formation of a population of ribosomes sufficiently large to last throughout the period of cleavage in the embryo.

A major difference between spermatogenesis and oogenesis is that spermatogenesis occurs after meiosis, whereas differentiation of the female gamete may occur early in meiosis.

During spermatogenesis, chromatin undergoes several dynamic transitions, which are often associated with important changes not only in its physical conformation but even in its composition and structure. In most instances, the compositional changes also alter the structure of chromatin [4]. As a result, chromatin becomes highly compacted and gene expression is completely shut down in the spermatozoon - a situation that totally differs from that encountered in oocytes, in which intense synthetic processes such as extra replication, transcription and RNP synthesis take place. The ways in which all these molecular events are achieved can be mediated by a wide spectrum of apparently diverse chromosomal proteins [5], which are mirrored by the morphological metamorphoses which accompany the formation of the mature sperm cell. The structure of chromatin conformation as a result of protein-DNA interactions in each particular situation is poorly understood in most instances, and the evolutionary relationship amongst these proteins remain obscure [6].

Our research was focused on structural and functional dynamics of chromatin during amphibian gametogenesis.

Materials and methods

Oocytes and testes from *Triturus cristatus* and *Xenopus laevis* were used in this study.

1) Electron microscopy technique. Testes were dissected and fixed in 2.5%-5% glutaraldehyde in 0.1 M sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in the same buffer for one hour at room temperature. They were then dehydrated in a graded ethanol series and embedded in Epon 812. The samples were sectioned on an ultramicrotome, stained in 4% aqueous uranyl acetate, post-stained with lead citrate and examined with a Philips 201 electron microscope.

2) Isolation of the lampbrush chromosome technique. A small bit of ovary was placed in a mixture of five parts 0.1 M KCl and one part 0.1 M NaCl. This mixture contains these two cations in proportion which was found within the oocytes nucleus. The cell was punctured with the forceps or a needle and squeezed gently. The nucleus appears as a clear “bubble” embedded in a ribbon of yolk flowing from the hole. In some instances, the nucleus was almost clean of yolk at this stage. As soon as the yolk has been removed from the surface of the nucleus, the latter was transferred to a flat-bottomed well slide, previously filled with chromosome isolation medium (five parts of 0.1 M KCl to one part of 0.1 M NaCl with formaldehyde added to a final concentration of 0.5%). The nuclear membrane was removed, and the contents of the nucleus have been spread. The slide has been covered with a coverslip, and examined with a phase-contrast microscope.

3) Fluorescence staining technique. After the spreading of nuclear contents, the samples were fixed with a mixture of 3 parts methanol and one part acetic acid, and after ten minutes washed and stained with acridine orange 0.01%. The samples were examined with Olympus fluorescence microscope.

4) Method for extraction of DNA from oocytes and sperm [7]. Each sample (100 mg of tissue) was suspended in microfuge tubes with 300 ml of digestion buffer (100 mM NaCl, 50 mM Tris-HCl, 1% SDS, 50 mM EDTA, pH 8.0). Proteinase K (10 mg/ml) was added to a final concentration of 100 mg/ml and the samples incubated for 2 h at 50°C, then overnight at 37°C. After digestion was complete, an equal volume of 5 M LiCl was added to each tube. The sample was mixed thoroughly by inversion for 1 min, then 600ml of chloroform was added and the samples were spun for 15 min at high speed (12000 rpm) in a bench-top microfuge and the supernatant was carefully removed to a new microfuge tube. Exactly 2 volumes of room temperature absolute ethanol were added and the tube inverted several times until the DNA precipitated. The samples were then resuspended in TE buffer. The samples were run in 0.8% agarose gel electrophoresis and using Bst E II I weight marker.

5) DNA digestion with isoschizomere endonucleases Msp I and Hpa II. After DNA purification, MspI, HpaII, enzyme buffer, BSA (stock concentration 5 mg/ml) and distilled water were added to a final volume of 45 ml. Each sample was incubated overnight at 37°C. The samples were run in 2 % agarose gel electrophoresis and using SMART weight marker.

Results and discussion

Using light (phase-contrast) and fluorescence microscopy it was possible to describe the organization of chromosomal lampbrush phenotype in oocytes and the accumulation of uncountable extrachromosomal nucleoli in their cytoplasm, as a result of ribosomal gene amplification phenomenon (Fig. 2, 3). Meiosis I is halted at the diplotene stage and the homologous chromosomes remain attached by chiasmata in bivalents. Each homologous chromosome in a bivalent is comprised of two parallel strands of sister chromatids. At intervals, the chromatin is compacted into chromomeres, and chromatin loops extended laterally from the chromomeres (Fig.1).

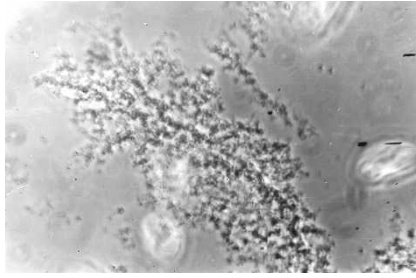


Fig. 1. Lampbrush chromosome from *Triturus cristatus* oocyte. The chromosome was isolated in saline solution and photographed with phase contrast microscope. It is obvious the bipartition of chromosomal axis and laterally extension of loops from the chromomeres. (X100).

Loops may have more than one transcription unit with opposite polarity [1]. The loops are quite active in transcription. Such loops exhibit clear asymmetry or a thickness gradient, being thin at one of their insertion points and as a result of the deposition of transcription products they become gradually thicker toward the other insertion point, which is adjacent to the first one. Considering the duration of the lampbrush phase, a tremendous amount of RNA can be synthesized during oogenesis in these organisms.

One strategy to produce more biological molecules in a short time is gene amplification by adopting of lampbrush chromosomal phenotype. The most obvious cytological marker of ribosomal gene amplification is the accumulation of extrachromosomal nucleoli in oocyte cytoplasm (Fig. 2, 3).

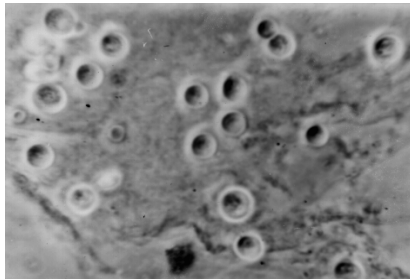


Fig. 2. *Triturus cristatus*. Phase-contrast optics view of extrachromosomal nucleoli isolated in saline solution (X100).

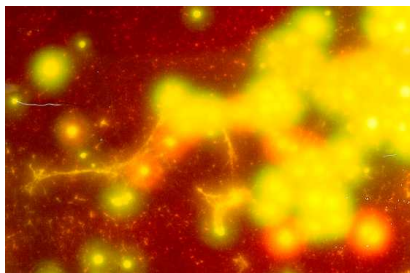


Fig. 3. *Triturus cristatus*. rDNA axial fiber connecting extrachromosomal nucleoli. Amplified nucleoli of newt oocytes arranged in characteristic disposition named "beaded rings"; the axial fiber that connects extrachromosomal nucleoli is rDNA. Acridine-orange staining, (red-RNA, yellow-green –proteins) (X100).

The nucleolus is the principal structure involved in the synthesis of high-molecular-weight rRNAs and the assembly of ribosomes. It has been estimated that with only the number of nucleoli normally found in cells, several hundred years would be required to produce the number of ribosomes found in the mature amphibian egg [8]. However, selective replication of those portions of the genome, which must account for the formation of ribosomes, reduces the time required to fulfill the production requirements of the oocyte to only a few months.

Ribosomal gene amplification was also studied at the molecular level. The electrophoretic study has shown that in the third and the fourth stages of oocyte development, additional bands corresponding to the oocyte amplified DNA are present (Fig. 4).

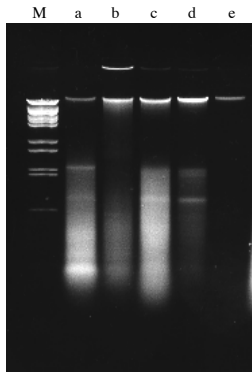


Fig. 4. Electrophoregram of DNA isolated from stages III and IV of oocytes development in *Xenopus laevis* (a, b), stages III and IV of oocytes development in *Triturus cristatus* (c, d), and from testes of *Triturus cristatus* (e); M-BstE II I molecular weight marker.

Ribosomal DNA amplification is not accomplished by the regular semi-conservative replication during S-phase replication of chromosomes. It proceeds by a “rolling circle” mechanism that is able to generate closed circles of DNA of widely variable length, thus accounting for the different length of beaded necklace nucleoli in oocyte nuclei. The result of gene amplification by rolling circle mechanism is the appearance of concatenare and concatemere structures. These structures have different molecular weight thus running across different lengths in agarose gel (Fig. 4).

Towards the end of the lampbrush phase, transcriptive activity would decline, polymerases would detach from loop axes, and loops would regress and disappear. In animals, the modified base 5-methylcytosine (5mC) is found predominantly in the sequence CpG [9]. Not all CpGs, however, are methylated. With the introduction of restriction endonucleases as probes for CpG methylation, it became possible to determine the relative locations of a subset of methylated and unmethylated CpGs along the DNA [10,11,12,13].

Ribosomal gene amplification and transcription are controlled by C residues methylation-demethylation processes. No 5mC was detectable in amplified rDNA [14].

DNA methylation dynamics in different stages of oocyte development, testes and liver tissue from *Triturus cristatus* was studied.

We have analyzed DNA restriction pattern from oocyte stages II, IV and VI of development with *MspI/HpaII* isoschizomere enzymes, and we found an unmethylated status in oocytes stage IV of development because the DNA is

digested to the same extent by MspI and Hpa II. This stage corresponds to the presence of the lampbrush phenotype which is involved in intense gene transcription processes, especially for ribosomal genes. DNA from testes is heavily methylated at HpaII site, gene expression is completely shut down in the spermatozoon – a situation that is totally different from that encountered in oocytes, in which intense synthetic processes such as extrareplication, transcription and RNP synthesis take place (Fig. 5).

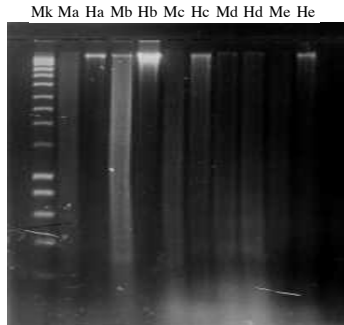


Fig. 5. Electrophoresis of DNA isolated from different types of tissues and then digested with restriction endonucleases MspI and HpaII in *Triturus cristatus* (Mk– molecular weight marker SMART; a- liver tissue, b- testes, c– stage II of oocytes development; d- stage IV of oocytes development; e- stage VI of oocytes development; M- Msp I; H - Hpa II).

Using TEM analysis, some unusual aspects of chromatin and chromosome organization and evolution in spermatogenesis of the amphibians *Triturus cristatus* and *Xenopus laevis*, were evidenced.

In a wide range of vertebrate organisms, the DNA within the sperm nucleus is organized into a genuine three-dimensional conformation due to replacement of histones with another class of nuclear proteins, namely *nuclear sperm-specific proteins*. Comparing the nucleosomal binding pattern of somatic histones with the binding pattern of nuclear sperm-specific proteins (that are localized in the minor groove of the DNA double helix) one can notice that nuclear sperm-specific proteins ensure an almost complete covering of the DNA, with very few uncovered areas. Thus, in contrast to the somatic cell nucleus, in the sperm nucleus the nucleosomal structure as well as the negative superhelicity are lost, the nucleoprotein complex acquiring a special physical conformation with very important functional consequences [15].

In the successive stages from spermatogonia to spermatozoa, we have deciphered the fine structural elements of cytoplasm and nuclear components (Fig. 6-8). The chromatids at the level of spermatids and spermatozoa achieve the highest condensation state of chromatin encountered in nature.

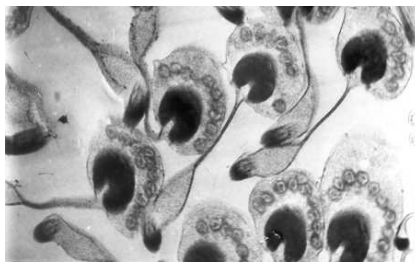


Fig. 6. *Triturus cristatus*. Transverse sections through a sperm head showing urodele features of a longitudinal juxta-axonemal fibre, a long undulating membrane, and mitochondria (X 23 214).

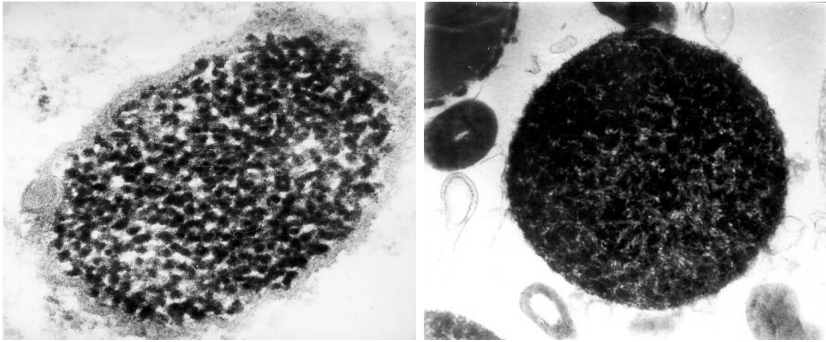


Fig. 7, 8. Intermediate stages of chromatin condensation in the transition from telophase II to spermatids, in *Xenopus laevis* (X 73 670).

As spermatids undergo the terminal stages of spermatogenesis, compacting the DNA requires the replacement of the histones with a class of arginine- and cysteine-rich proteins called protamines. Although the reason for replacement of the histones with protamines is unknown, one possibility is the generation of a more hydrodynamic sperm head that speeds the transit through the female reproductive tract and across the zona pellucida surrounding the egg. It may also be that the nucleoprotamine structure protects the genetic material in the sperm head from physical and chemical damage. Alternatively, packing of sperm chromatin may serve to reprogram the paternal genome so that the appropriate genes from the male progenitor's chromosomes are expressed in the early embryo.

Displacement of histones by transition proteins and protamines is accompanied by several post-translational modifications: histone acetylation (especially histone H4 acetylation), ubiquitination and phosphorylation all facilitate the displacement of histones [16]. Chromatin remodeling may also require chaperones that actively displace the post-translationally modified histones [17]. Because of the gradual replacement of histones, intermediate stages of chromatin condensation are evidenced (Fig. 7, 8).

In many sections of *Xenopus* spermatocyte nuclei, the presence of bivalents consisting of homologous chromosomes joined by synaptonemal complexes could be evidenced (Fig. 9).

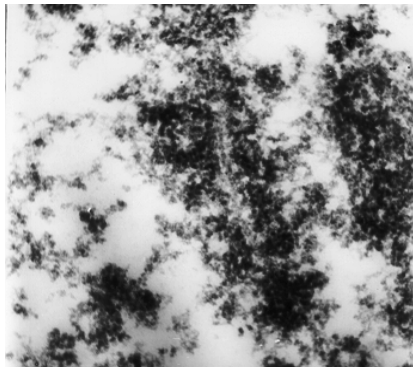


Fig. 9. Spermatocyte nucleus of *Xenopus laevis*: bivalents with homologous chromosomes held together by synaptonemal complexes. (X 49 764).

Conclusions

The study of nuclear molecular architecture and chromosomal phenotype during gametogenesis represents one approach towards deciphering the molecular organization of the eukaryotic chromosome in relation with their functional activities.

Why do lampbrush chromosomes exist at all? They are characteristic of eggs that develop rather quickly into complex multicellular organisms independently of the parent. A frog's egg, for example, is fertilized, deposited by the mother and then develops into a complex tadpole within a few days. Lampbrushes may therefore be regarded mainly as an adaptive feature that has evolved to preprogram the egg for rapid early development. One strategy to produce more biological molecules in a short time is gene amplification through adopting the lampbrush chromosomal phenotype. The most obvious cytological marker of ribosomal gene amplification is the accumulation of extrachromosomal nucleoli in oocyte cytoplasm.

During spermiogenesis there is a dramatic remodeling of chromatin that is characterized by a considerable cellular morphological change, concurrent with modifications in the nature and content of the nuclear basic proteins. The chromatin of the mature sperm adopts in the majority of instances a highly compacted state in which gene expression is completely repressed. Spermatogenesis is a complex differentiation process which is characterized, among other features by conspicuous stage-specific nuclear events such as the pairing of homologous chromosomes coupled with the formation of synaptonemal complexes, the replacement of histones with sperm-specific proteins during spermiogenesis and, as a result, chromatin condensation and its inactivation in sperm cells. TEM analysis has revealed that the chromatin of spermatogenic cells undergoes structural rearrangements upon differentiation from spermatogonia to mature spermatozoa.

Developmental reprogramming of the parental genomes occurs during egg and sperm formation. However, the direct relationship between chromatin repackaging in sperm and developmental reprogramming is unknown.

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