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Chromatin dynamics in *Triturus cristatus* oogenesis: an epigenetic approach

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Summary

Oogenesis is a critical event in the formation of female gametes, whose role in development is to transfer genomic information to the next generation. During this process, the gene expression pattern changes dramatically concomitant with genome remodelling, while genomic information is stably maintained. The aim of the present study was to investigate the chromatin architecture in newt oocytes. Using fluorescence microscopy, as well as transmission electron microscopy (TEM), immunohistochemical method and RE-ChIP assay, some peculiar aspects of chromatin and chromosome organization and evolution in crested newt oogenesis were investigated. We focussed our investigations on detection of certain epigenetic modifications (H4 hyperacetylation, H2A ubiquitinylation and cytosine methylation) at the rRNA gene (18S–5.8S–28S) promoter region. Our findings suggest that there is an involvement of some epigenetic modifications as well as of linker histone variants in chromatin architecture dynamics during crested newt oogenesis.

Keywords: Chromatin remodelling, Epigenetic modifications, Linker histones, Oogenesis, rRNA gene promoter

Introduction

Germinal cells play key roles in heredity, the differentiation of these cells requires complex and specific structural architecture dynamics of their genetic material.

One particular aspect of amphibian oogenesis is the formation of the lampbrush chromosomal phenotype during the major phase of oocyte growth and differentiation (Macgregor, 1993). Lampbrush chromosomes are considered uniquely valuable tools in the study of development of their two major characteristics: (i) they are transient structures that are formed during an prolonged diplotene of the first meiotic division; and (ii) their most functional conspicuous feature is widespread RNA transcription, which take place on hundreds, and in some cases thousands, of loops that are laterally projected from the main axis of the bivalent at short intervals along its entire length. Therefore, lampbrush chromosomes are good experimental models for the study of chromosome organization and gene expression during meiotic prophase (Macgregor, 1993).

The early diplotene nucleus is also characterized by the formation of a large number of extrachromosomal nucleoli (up to 1500, in *Xenopus*), which further are distributed beneath the nuclear membrane (Barsacchi *et al.*, 1970; Steopoe *et al.*, 1985). These nucleoli are the morphological expression of a phenomenon known as specific ribosomal gene amplification (Brown & Dawid, 1970), an event controlled by certain chromatin changes that trigger local decondensation and remodelling in order for the nuclear machinery to progress through the signalling and transcription processes (Grant, 2001). An emerging theme in the field of chromatin research has relied on the role of histone post-translational modifications in nuclear function regulation.

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One of the most important landmarks in the chromatin field during the last two decades has been the dynamic view, which states that histones are not merely passive structural players but rather they have an important functional role in the modulation of gene expression (Grunstein, 1990; Wolfe, 1992). Research over the past decade has revealed that their chromatin structure is far more than a static carrier of the genetic information encoded in their DNA, as it actively and dynamically mediates regulation of processes that require protein access to DNA, i.e. transcription, recombination and replication. Emerging evidence indicates that epigenetic factors control and regulate most nuclear processes. These factors alter chromatin structure by covalent DNA modifications, covalent histone modifications and nucleosome repatterning. These changes seem to be interpreted by proteins that recognize a particular modification and facilitate the appropriate downstream biological effect (Turner, 1991, 1993; Ausio, 1992; Bird, 1996; Strahl & Allis, 2000; Jenuwein & Allis, 2001; Lusser, 2002; Jaenish & Bird, 2003; Santoro, 2005).

The nucleolus, the site of ribosomal precursor synthesis and ribosome assembly, is an example of an essential nuclear organelle (Peng & Karpen, 2007). In somatic cells, the nucleolar organizing region (NOR) contains tandemly repeated ribosomal RNA genes (rDNA), and is embedded in heterochromatin in most eukaryotes. Single rDNA genes inserted into euchromatin are able to form mini-nucleoli through a self-assembly process that is probably initiated by rDNA transcription (Karpen *et al.*, 1998). The genes that encode rRNA are tandemly repeated at one or few chromosomal loci in eukaryotes. The number of rRNA gene repeats varies greatly among organisms, ranging from less than 100 to more than 10 000 (Long & Dawid, 1980). One might imagine that the presence of large numbers of rRNA gene copies reflects a demand for higher levels of rRNA synthesis during rapid growth of early embryo. In somatic cells only a fraction of these repeats is used for rRNA synthesis at any given time. In metabolically active human or mouse cells, approximately half of the 400 rRNA gene copies are transcriptionally active, and the other half are silent (Conconi *et al.*, 1989; Santoro *et al.*, 2002; Lawrence *et al.*, 2004).

Active and silent genes are distinct from one another with respect to their chromatin configurations; active genes are located in euchromatin, whereas silent genes are usually located in heterochromatin (Santoro *et al.*, 2002). Recent studies have established that epigenetic mechanisms are involved in marking the transcriptional state of rRNA genes in human and mouse somatic cells, which is transmitted to daughter cells in order to assure a correct chromatin conformation dynamics. The promoter of active rRNA genes is free of CpG methylation and is associated with acetylated histones, an opposite pattern being predominant among silent genes (Lawrence *et al.*, 2004). The epigenetic mechanism of rRNA gene silencing was extensively studied for the nucleolar dominance phenomenon (Reeder, 1985; Lawrence *et al.*, 2004; Santoro & Grummt, 2005; McStay, 2006), which is common in plant and animal hybrids, whereby rRNA genes inherited by one parent are transcribed and those from the other parent are silent.

In contrast to rRNA genes from somatic cells, epigenetic networks that mediate transcriptional state of rDNA from gametes have been poorly studied. The aim of this study was to investigate amphibian oocyte chromatin remodelling process from genome level to 28S–5.8S–18S ribosomal gene promoter. This approach (using complex methodology that included a variation on the chromatin immunoprecipitation assay (RE-ChIP), a transmission electron microscopy technique, immunohistochemistry and the electrophoretic analysis of histones) explained a relationship between chromatin structure and DNA function, thereby suggesting certain aspects of epigenetic regulation of the gene expression and molecular signalling during oogenesis.

Materials and methods

Animals

Female newts (*Triturus cristatus*) were obtained from Bucharest Botanical Garden Pool, University of Bucharest. Animals were sacrificed in accordance with stipulations of European Council law (86/609/CEE/24.11.2004) regarding the protection of animals used for experimental and scientific aims.

Acridine orange (AO) staining of oocyte nucleoli

A small piece of ovary was placed in a mixture of five parts 0.1 M KCl and one part 0.1M NaCl. This mixture contained these two cations in the ratio that was found within the oocytes nucleus. The cell was enucleated and the nucleus was transferred to a flat-bottomed well slide, previously filled with chromosome isolation medium (five parts 0.1 M KCl to one part 0.1M NaCl, with formaldehyde added to a final concentration of 0.5%). After spreading the nuclear contents, the samples were fixed with a mixture of methanol and acetic acid 3 : 1 (v/v), and after 10 min washed and stained with AO 0.01%. Acridine orange interacts with DNA and RNA by intercalation or electrostatic attraction respectively. DNA-intercalated AO fluoresces green and RNA electrostatically bound to AO fluoresces red. The samples were examined with an Olympus fluorescence microscope.

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Electron microscopy

Vitellogenic oocytes were collected and enucleated directly in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Electron microscopy technique was performed as described previously (Burlibaşa & Gavrilă, 2005). The samples were examined with a Philips 201 electron microscope.

Extraction of oocyte histones

For extraction of histones from previtellogenic and vitellogenic oocytes of *T. cristatus*, frozen oocytes from a single animal were resuspended in approximately 0.5 ml of 0.15 M NaCl, 10 mM Tris–HCl pH 7.5 buffer containing 0.2 mM phenylmethylsulfonylfluoride (PMSF). The samples were then disrupted in this buffer using an homogeniser. The homogenate was spun down for 10 min at 16 000 *g* at 4 °C, in a centrifuge. The pellets were re-suspended in buffer (0.5 ml 0.15 M NaCl, 10 mM Tris–HCl, 0.2 mM PMSF, 0.5% Triton X-100) and were homogenized and centrifuged for 10 min at 16 000 *g* at 4 °C. This step was usually repeated twice. The final pellets were resuspended in approximately 0.8 ml of 0.4 N HCl, homogenized and kept at 4 °C with occasional stirring. The HCl extracts were next spun down at 16 000 *g* in the Eppendorf microfuge for 10 min. The supernatants thus obtained were then precipitated with 5 volumes of acetone for 1–2 h at –20 °C. The tubes were centrifuged using the above procedure and the pellets were dried and then re-suspended in distilled water.

Electrophoretic fractionation of histones

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12.5% slab gels, pH 6.8, with 1.5 M Tris–HCl running buffer, pH 8.8, according to the Laemmli system. Unfractionated histones (1 mg/ ml) were dissolved in the tray buffer, which contained 0.125 M Tris–HCl, 4% SDS, 20% v/v glycerol, 0.2 M DTT and 0.02% bromophenol blue, pH 6.8. The gel was run at a constant voltage 10 V/cm. After electrophoresis, gels were stained with 0.025% (w/v) Coomassie blue in 40% (v/v) methanol/7% (v/v) acetic acid and distained in 40% (v/v) methanol/7% acetic acid (v/v). Histones were fractionated comparatively with a lysine-rich histone marker (f1 fraction – Sigma), an arginine-rich histone fraction (f3 fraction – Sigma) and a molecular weight marker (Sigma) ranging between 11.2 kDa and 66 kDa. The gels were scanned with Jencons-PLS UVB Bioimaging System.

Immunohistochemistry

Acetylated histone H4 labelling was performed using rabbit polyclonal antibody against the penta-acetylated peptide corresponding to histone H4 (Upstate Biotechnology). Fragments of newt ovary were fixed in Bouin solution dehydrated in ethanol, cleared in toluene and embedded in paraffin. Immunohistochemistry was performed on 7-μm thick sections as described previously (Zarnescu, 2007).

Chromatin immunoprecipitation (ChIP) and RE-ChIP assays

The following antibodies were used in this study: (1) rabbit anti-histone H4, hyperacetylated (Penta) polyclonal antibody (Upstate Biotechnology NY); (2) anti-ubiquityl-histone H2A, clone E6C5, mouse IgM, (Upstate Biotechnology NY); and (3) anti-5′-methyl cytosine, sheep IgG (Biomol).

ChIP studies were performed using a Quick ChIP Kit (Imgenex) according to a variant adapted by us from the Quick ChIP Kit user manual. All the steps were performed at 4 °C. Immature (previtellogenic) and vitellogenic oocytes (defolliculated previously) were incubated for 10 min with 1% formaldehyde by gentle agitation. Cross-linking was stopped by the addition of 0.125 M glycine for 10 min with gentle agitation at room temperature. The cells were then washed with phosphate-buffered saline (PBS), scrapped off in PBS and centrifugated at 1000 *g* for 5 min. The cell pellet was resuspended in lysis buffer containing SDS, a mixture of proteinase inhibitors (10× PIC) (Quick ChIP kit) and 10× PMSF and then further incubated for 10 min on ice. The cell extract was collected by centrifugation at 1000 *g* for 10 min, resuspended in washing buffer A and homogenized with an equal volume of washing buffer B. The mixture was then centrifuged at 1000 *g* for 15 min. The cell pellet was resuspended in NUC buffer (15 mM HEPES pH 7.5, 60 mM KCl, 15 mM NaCl, 0.13 mM sucrose, 0.15 mM β-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine) and then $CaCl_2$ (3 mM final concentration) and 50 UI/ml MNase (micrococcal nuclease) (Biomol) were added. The reaction was stopped with an equal volume of Buffer X (90 mM HEPES pH 7.9, 220 mM NaCl, 10 mM EDTA, 2% Triton X-100, 0.2% SDS, 0.5 mM PMSF) containing 10 μl 10× PIC. After centrifugation at 16 000 *g*, the supernatant was collected and mixed with dilution buffer and 10 μl of 10× PIC (Quick ChIP kit). An aliquot was used for A260 measurements. Cross-linked extracts (10 A260 units) were resuspended in 75 μl salmon sperm DNA/protein G Agarose (Quick ChIP kit), early blocked with bovine serum albumin (BSA) (Sigma) and were incubated for 30 min at 4 °C by agitation. After centrifugation at 1000 *g* for 2 min, the supernatant was collected and aliquots of 10 μl were stored at 4 °C. After centrifugation at 1000 *g* for 2 min, the supernatant was

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collected and immunoprecipitated with anti-histone H4 hyperacetylated (Upstate Biotechnology) by mixing overnight at 4 °C. The immunocomplexes were recovered with the addition of 60 μl salmon sperm DNA/protein G agarose and subsequent incubation for 1 h at 4 °C with agitation. The complexes were washed with buffer A and centrifuged at 1000 *g* for 1 min. The pellets were resuspended and washed with buffer B, centrifuged and the procedure repeated for washing with buffer C (Quick ChIP kit) and buffer D (Quick ChIP kit). The protein–DNA complexes were then eluted by incubation with 250 μl of elution buffer (50 mM $NaHCO_3$ and 1% SDS) for 15 min at 65 °C. The beads were incubated with an equal volume of 10 mM DTT for 30 min at 37 °C, and then centrifuged at 1200 *g* for 1 min and the supernatant was transferred into a new tube. The samples were diluted 40 times with elution buffer and 10% of the sample was kept as an input sample (positive control for first antigen presence) and processed by adding the second antibody, anti-ubiquityl-histone H2A, clone E6C5, respectively anti-5′-methyl cytosine. The protein–DNA complexes were then eluted by incubation in 250 μl of elution buffer (50 mM $NaHCO_3$ and 1% SDS) for 15 min at 65 °C, then centrifuged at 1200 *g* for 1 min and the supernatant was transferred into a new tube. After centrifugation at 1000 *g* for 5 min, the supernatant was collected and incubated with 10 μg/ml RNase A for 1 h at 42 °C. Then NaCl was added to mixture to a final concentration of 200 mM and incubated at 65 °C to reverse cross-linking. The proteins were then digested with 200 μg/ml proteinase K for 2 h at 50 °C. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation. PCR amplification of a region of 544 bp from ribosomal genes promoter −85 to +395 has been performed using primers having sequences chosen from NCBI accession numbers X02995 and X98876 (forward 5′-ATTAAGGTCCGCCGGACT-3′ and reverse 5′-GCCCTCGCTCGGATAAAA-3′) and Ampli*Taq* polymerase (Biomol), in a Perkin Elmer thermocycler. The following PCR programme was set up: initial denaturation 94 °C for 5 min, 25 cycles × (94 °C/60 s, 53 °C/60 s, 72 °C/60 s) and final elongation for 7 min at 72 °C. Two control samples: positive control (input) sample – for the presence of H4 hyperacetylated and negative control sample were run through each step of the ChIP procedure without antibody. PCR products were run on an agarose 1% gel.

Results

Acridine orange staining of vitellogenic oocyte nucleoli

The multinucleolar state of the vitellogenic oocytes nucleus was evidenced by spreading isolated nuclei using a saline medium technique and acridine orange staining. The nucleolar organizer region (NOR) is the main structure that is involved in the synthesis of high-molecular-weight rRNAs and in the assembly of ribosomes. Different nucleoli have different fluorescent colours in accordance with their composition (DNA – yellow/green; RNA – red/orange). Fluorescent staining revealed a 'string of beads' structural pattern for the extrachromosomal nucleoli (Fig 1).

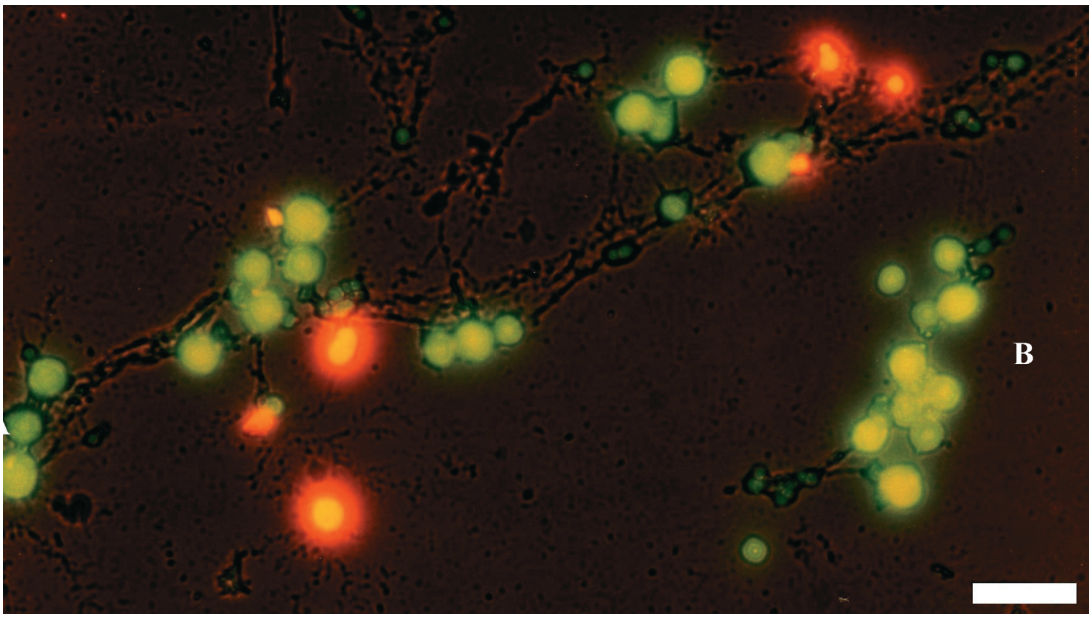

Figure 1 Acridine orange staining of nucleoli isolated in saline solution from *Triturus cristatus* fully grown oocytes (red – RNA, yellow – proteins). Extrachromosomal nucleoli of newt oocytes are arranged in 'beaded rings' (bar: 5 μm).

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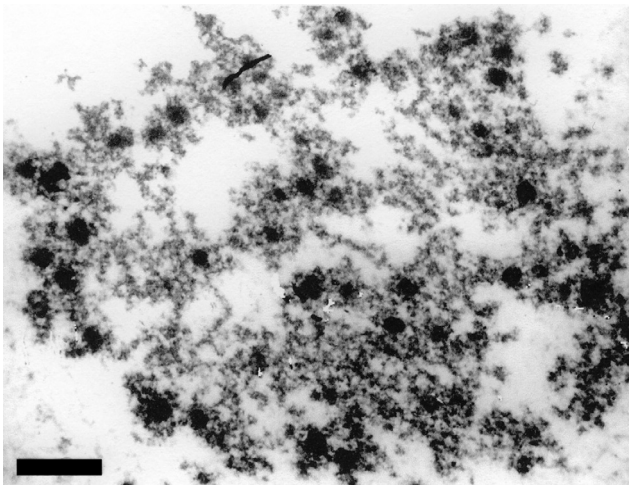

Figure 2 Electron micrograph of a nucleus from *Triturus cristatus* oocyte. A putative folded transcriptionally active lampbrush loop is evidenced. Multiple RNP particles are randomly distributed over almost whole length of the lampbrush loop (bar: 0.5 μm).

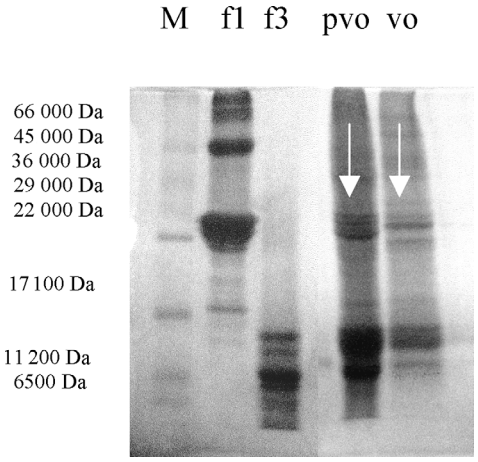

Figure 3 *Triturus cristatus*: 12.5% SDS-polyacrylamide gel electrophoresis of histones from previtellogenic (pvo) and vitellogenic (vo) oocytes. M, molecular weight marker; f1, lysine rich histone marker (H1); f3, arginine rich histone fraction (nucleosomal core histones). The electrophoregram indicates the presence of four variants of linker histone H1 (pvo line) in previtellogenic oocytes and two variants of linker histone (vo line) in vitellogenic oocytes.

Ultrastructural analysis of a lampbrush loop

We have studied electronmicroscopically nuclei that were isolated from vitellogenic oocytes. The multiple ribonucleoproteins (RNP) complexes are distributed at the lampbrush loops (Fig. 2). Fine sections were visualized using TEM, these showed that the lateral loops are actively transcribed and their profiles are associated with the richness of RNP particles, representing the transcription products.

Electrophoretic fractionation of previtellogenic and vitellogenic oocytes histones

Previous literature had suggested that linker histones and their variants might play a specific role in transcription regulation (Zlatanova, 1990), therefore the histone content pattern during oogenesis was studied. Histones from previtellogenic and vitellogenic oocytes were isolated and fractionated.

The electrophoregram indicated the presence of nucleosomal core histones (H2A, H2B, H3 and H4), both in previtellogenic and vitellogenic oocytes, and were seen as four bands that exhibited the same electrophoretic mobility as the f3 fraction (Fig. 3).

In previtellogenic oocytes, the electrophoretic pattern indicated the presence of four variants of H1 histone (pvo line), whereas, for vitellogenic oocytes, isolated histones indicated the presence of two variants of H1 histone (vo line). These results suggest a clear variation in the linker histone pattern in previtellogenic and vitellogenic oocytes. Stages III and IV of vitellogenic oocytes are cytological correspondents of the lampbrush chromosome phenotype involved in amplification of the ribosomal genes and of their high transcriptional activity.

Immunohistochemical investigations of the hyperacetylation of histone H4 in vitellogenic oocytes

The aim of the next set of experiments was to investigate the relationships between H4 hyperacetylation and the specific regions of transcriptional activity within chromatin of newt oocytes. A study of the immunohistochemical distribution of H4 hyperacetylated in vitellogenic oocytes (stages II–IV) and oocyte stages, correlated with the lampbrush phenotype, was performed.

Immunodetection of hyperacetylated histone H4 (tri- and tetra-acetylated forms of histone H4) on histological preparations of crested newt ovary revealed a characteristic nuclear staining.

In the oocyte nucleus there are numerous extrachromosomal nucleoli with preferential distribution at the nuclear periphery (Fig 4*a*, *b*). From such extrachromosomal multiple nucleoli, smaller nucleolar bodies are released and that are ready to pass through the nuclear envelope into the cytoplasm (Fig. 4*c*).

Immunoperoxidase staining of hyperacetylated H4 resulted in a homogeneous nuclear pattern with a massive concentration of signals for hyperacetylated H4 at the periphery of the nucleus, in close vicinity to the internal surface of the nuclear envelope (Fig. 4*b*, *c*). These peripheric blocks showed a high immunoreactivity for hyperacetylated H4. In

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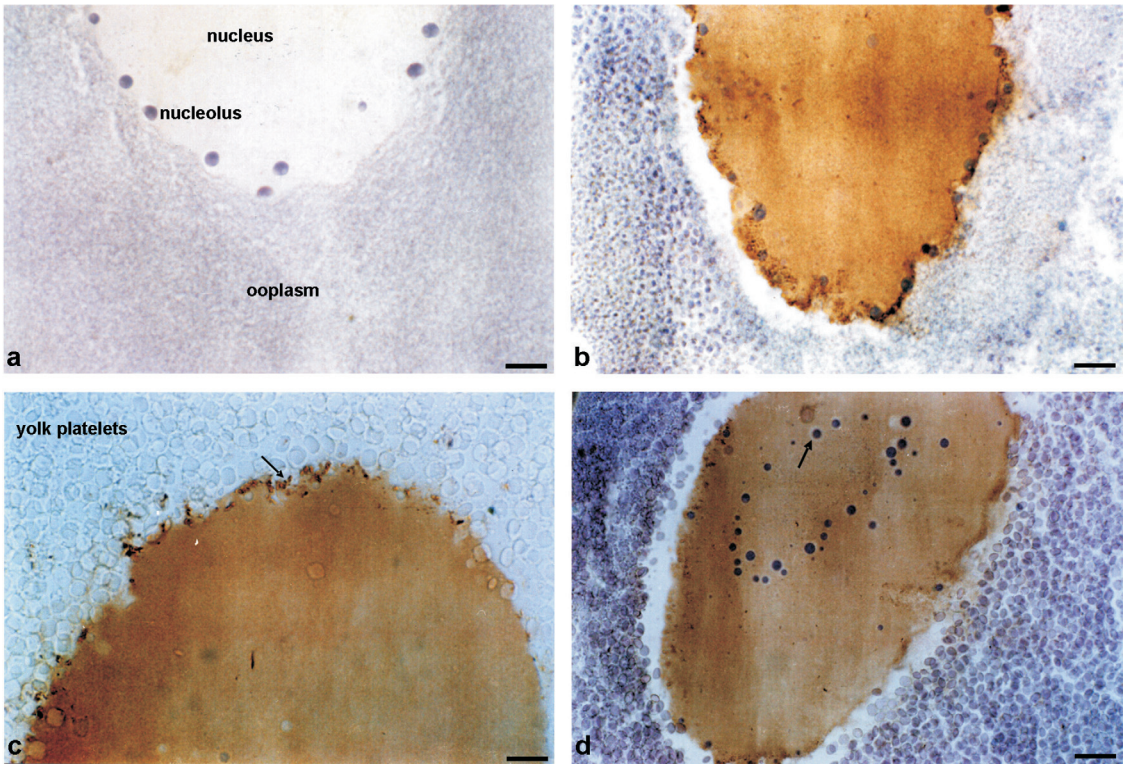

Figure 4 Immunohistochemistry for hyperacetylated H4 from vitellogenic oocytes of *Triturus cristatus* (*a*, *b*, *d* – counterstained with Harris' haematoxylin). (*a*) Negative control for immunohistochemical reactions; early vitellogenic oocytes; numerous extrachromosomal nucleoli with a preferential distribution at the periphery of the nucleus are evidenced. (*c*–*d*) Immunostaining for H4 hyperacetylated revealed a homogeneous nucleoplasm pattern with a massive concentration of signals for highly acetylated H4 at the periphery of the nucleus. (*b*, *c*) Some peripheral blocks of hyperacetylated H4 are detached and the invagination of the nuclear membrane becomes evident. (*d*) Nucleoli exhibit a characteristic form as 'beaded rings'. Immunostaining was lacking in extrachromosomal nucleoli and in some perinucleolar regions (bars: 25 μm).

some sections, multiple extrachromosomal nucleoli exhibited a characteristic form known as 'beaded rings' (Fig. 4*d*). Immunostaining failed in multiple extrachromosomal nucleoli. Another peculiar aspect of the immunostaining pattern was the lack of a signal for highly acetylated isoforms of histone H4 in certain perinucleolar regions (Fig. 4*d*).

In Fig. 4*c*, a detachment at the level of these peripheral blocks of hyperacetylated H4 *histone* to the oocyte cytoplasm is evident. Interestingly, this event is mediated by the detachment of the H4 hyperacetylated blocks and concomitant nuclear envelope invagination.

RE-ChIP assay for studying the epigenetic modifications at the rRNA gene promoter

To our knowledge the epigenetic modifications at the level of ribosomal genes promoter in amphibian oocytes has not been reported previously. For this approach, we considered it appropriate to analyse the changes in chromatin structure at the promoter region of ribosomal cistron employing the methods for the detection of certain epigenetic modifications (H4 hyperacetylation, H2A-ubiquitination and cytosine methylation).

In order to investigate the epigenetic repertoire at the level of ribosomal cistron promoter, a variation on the immunoprecipitation ChIP assay, RE-ChIP, was used.

The ChIP assay was employed to evaluate the association of histone and non-histone proteins with a specific DNA region. This technique involves crosslinking of proteins with DNA, fragmentation and preparation of soluble chromatin, followed by immunoprecipitation with an antibody that recognizes the protein of interest. The segment of the genome that is associated with the protein is then identified by PCR amplification of the DNA in the immunoprecipitate. Re-ChIP involves sequential chromatin immunoprecipitations with two antibodies in order to study the simultaneous presence of two proteins or different histone modifications in the genome sequence of interest.

Because ribosomal genes are organized in tandem repeats (i.e. many copies of ribosomal genes exist in a genome) and in order to study the simultaneous presence of two epigenetic modifications at the same

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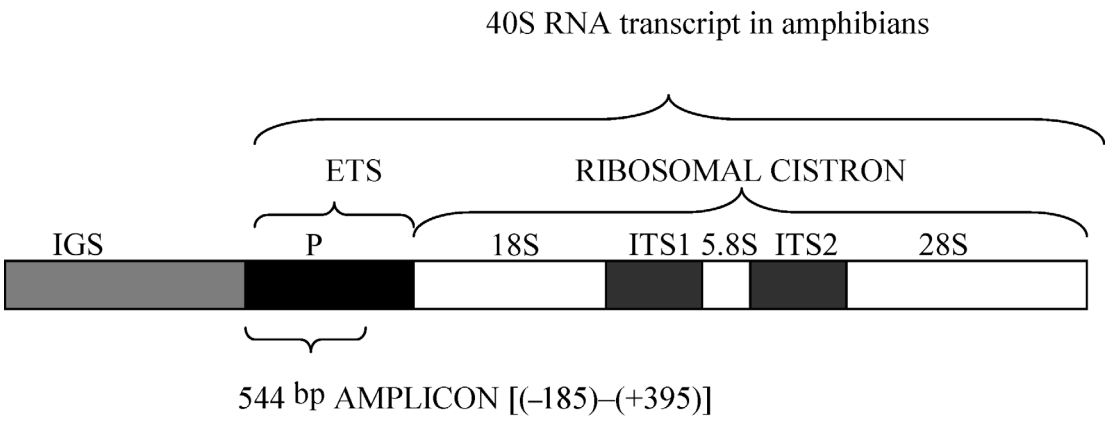

Figure 5 Amphibian ribosomal cistron organization. ITS, internal transcribed spacer; ETS, external transcribed spacer; IGS, intergenic spacer; P, promoter.

promotor region, this variation of the ChIP assay was used. In this study, the segment of genome analysed for association with certain epigenetic modifications (H4 hyperacetylation, H2A-ubiquitination and cytosine methylation) is represented by a sequence found 544 bp from the ribosomal cistron (18S–5.8S–28) promoter. The amplicon also contains a small fragment from the external transcribed spacer (ETS) (Fig. 5). The amphibian rRNA gene promotor is an oligo dT promotor that contains a conserved 5′-GCTTTTTTG-3′ sequence.

In order to ensure accurate results in the RE-ChIP assays, chromatin was fragmented by MNase incubation for several time periods, thus obtaining fragments with a range of 300–1000 bp. We tested four different MNase (50 UI/ml) incubation times (5, 10, 15 and 30 min) for both previtellogenic and vitellogenic oocytes. We choose the length of chromatin fragments to be less then 1000 bp (confirmed by electrophoretic analysis) at 15 min MNase incubation time for previtellogenic oocytes and, respectively, we choose a 10 min MNase incubation time for vitellogenic oocytes (Fig. 6). The electrophoresis pattern indicates different levels of chromatin sensitivity to MNase in previtellogenic and vitellogenic oocytes depending on its level of condensation.

In the first RE-ChIP experiment (hacH4 + 5MeC), the electrophoregram indicated the simultaneous presence of both epigenetic modifications (high acetylated H4 forms and 5′-methyl cytosine) in previtellogenic oocytes at equivalent levels in the rRNA promoter region. For vitellogenic oocytes, only highly acetylated H4 forms were present. The Re-ChIP procedure, combined with PCR analysis, showed a negative result for cytosine methylation for the sequence of interest (Fig. 7*a*).

The second experiment was designated to study the simultaneous presence of high acetylated forms of histone H4 and ubiquitinylation of H2A (hacH4 + uH2A), using the same comparative study of a negative control and positive (input) sample for hyperacetylated H4.

Interestingly, PCR analysis gave a positive result for the RE-ChIP assay in previtellogenic oocytes and a negative result for both antibody immunoprecipitations

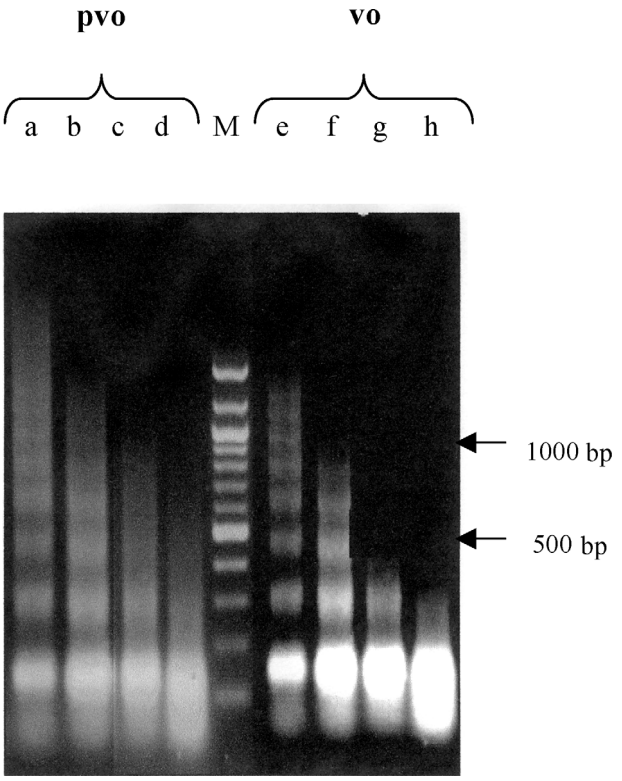

Figure 6 Micrococcal nuclease digest (50 UI/ml) of the chromatin from previtellogenic (pvo) and vitellogenic (vo) oocytes. Various MNase incubation times were tested (*a*) 5 min; (*b*) 10 min; (*c*) 15 min; (*d*) 30 min; (*e*) 5 min; (*f*) 10 min; (*g*) 15 min; and (*h*) 30 min). Samples (*c*) and (*f*) were used in RE-ChIP experiments (M, marker 100 bp, Roth).

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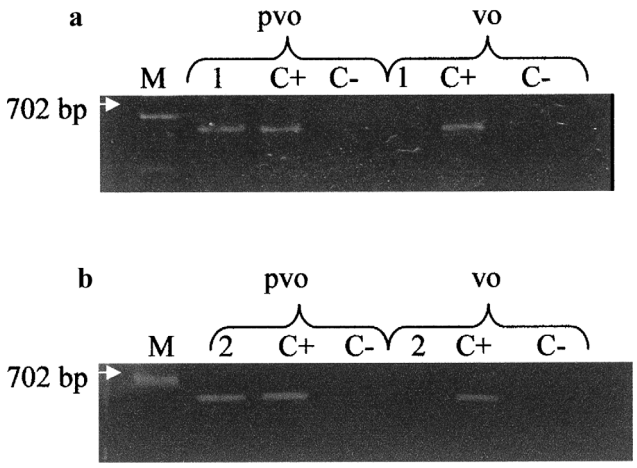

Figure 7 RE-ChIP experiments. (*a*) PCR analysis of hacH4 + 5MeC immunoprecipitates from previtellogenic (pvo) and vitellogenic (vo) oocytes. M, molecular weight marker BstE II λ; 1, hacH4 + 5MeC, (C^+), positive control (input sample); (C^-), negative control (without antibodies); (*b*) PCR analysis of hacH4 + uH2A immunoprecipitates from previtellogenic (pvo) and vitellogenic (vo) oocytes. 2, hacH4 + uH2A; (C^+), positive control (input sample); (C^-), negative control (without antibodies).

in vitellogenic oocytes (Fig. 7*b*). These results indicate the simultaneous presence of highly acetylated H4 and ubiquityl-H2A at the ribosomal gene promoter region, in previtellogenic oocytes. For vitellogenic oocytes only highly acetylated forms of histone H4 were detected.

Taken together, our results suggest that previtellogenic oocytes have a different repertoire of epigenetic modifications at the level of the ribosomal gene promoter region as compared with vitellogenic oocytes.

Discussion

During amphibian oocytes development, chromatin remodelling factors act towards the decondensation of chromatin fibre; this process enables the access of transcription factors or signalling molecules to DNA embedded in the chromatin structure. Chromosomes that adopt a lampbrush phenotype are involved in extensive transcription activity. Chromatin structure plays an important role in the control of gene expression by limiting the accessibility of sequence-specific binding proteins to DNA. The histone components of chromatin play an integral role in this regulation machinery. Several years ago it was suggested that linker histones may play a more specific role in transcription regulation by acting at the level of critically placed individual nucleosomes, rather than by fibre compaction generally (Zlatanova, 1990; Brown *et al.*, 1997; Gunjan & Brown, 1999). H1 was often supposed to function as a general repressor of transcription by stabilizing higher order chromatin structure (Carruthers *et al.*, 1998). Linker histones have also been demonstrated to reject factor binding directly (Juan *et al.*, 1994), in order to limit nucleosome mobility (Ura *et al.*, 1995) and to reduce the transient dynamic exposure of DNA on the nucleosome surface (Polach & Widom, 1995). All of these activities are expected to have repressive effects on transcription. The fact that linker histones are actually involved in transcriptional regulation of individual genes was first shown unambiguously in two systems: (i) the selective transcription of oocyte- and somatic-type 5S rRNA genes in early *Xenopus* development (Kandolf, 1994); and (ii) the activation and repression of transcription of certain genes in *Tetrahymena* knockouts for histone H1 (Shen *et al.*, 1996).

In our study, 12.5% SDS-PAGE electrophoresis results regarding linker histones from previtellogenic and vitellogenic oocytes revealed an interesting dynamics of chromatin H1 variant content during oocyte development. Previtellogenic oocytes were shown to contain more linker histone variants than mature oocytes, the latter being involved in an extensive transcriptional activity. These results suggest a hypothesis in which oocyte differentiation is marked by a gene expression regulation process through specific dynamics of histone H1 variants. Moreover, we also suggest that these H1 variants have a functional significance during amphibian oocytes differentiation.

Most of our knowledge of transcriptional regulation comes from studies in somatic cell, in which specific chromatin remodelling events are directly coupled with transcriptional activation and silencing (Jenuwein *et al.*, 2001). One can ask: 'Are the same events operating in egg cells?'

Oogenesis is a critical event in the formation of female gametes, highly specialized cells that transfer the maternal genomic information to the next generation. During this process, the gene expression pattern changes dramatically, concomitant to genome remodelling, while genomic information is maintained. There are also many meiosis-specific events other than the reprogramming of gene expression in oocytes, e.g., homologous chromosomes recombination and successive divisions without DNA replication, asymmetric cell division. All these events are accompanied by meiotic specific modifications in chromatin structure and some of these might be controlled by epigenetic modifications including histone acetylation and DNA methylation.

One of the extensively studied epigenetic modification is histone acetylation. The acetylation of nucleosome core histones is thought to play an important role in various functions. The biological significance of histone acetylation was first suggested

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after the discovery that the amount of acetylation is correlated with the level of transcription (Allfrey *et al.*, 1964). At the beginning of this century, the reversible acetylation of core histones had been recognized as a major mechanism by which chromatin-mediated gene regulation is affected (Gunjan *et al.*, 2001). Some reports provide direct evidence that the acetylation status of core histones has a causal relationship to gene activity (Wang *et al.*, 1998). It has been demonstrated that many of the histone-acetyltransferases (HATs) that acetylate core histones are components of transcriptional activator or coactivator complexes and are specifically targeted to genes in order to activate transcription (Utley *et al.*, 1998). On the other hand, transcriptionally silent chromatin is often hypo-acetylated and histone-deacetylases (HDACs) have been shown to be components of transcriptional corepressors and silencers that are also targeted to the appropriate DNA sequences (Ayer, 1999).

Certain studies on H4 acetylation (Sommerville *et al.*, 1993; Kim *et al.*, 2003) and its turnover (Magnaghi-Jaulin & Jaulin, 2006) in oocyte chromatin have been reported previously. Lampbrush chromosomes from previtellogenic (immature) and vitellogenic (mature) oocytes of the amphibian *Triturus cristatus* have been used to examine the role of histone acetylation in transcription by indirect immunofluorescence with antisera to H4 that was acetylated at specific lysine residues (lysines 5, 8, 12 and 16) (Sommerville *et al.*, 1993). This work suggested that H4 acetyl groups turn over more rapidly in mature than in immature oocytes and that histone hyperacetylation proceeds, and possibly induces, loops formation and transcriptional activation.

Our immunohistochemical investigations on the hyperacetylation of the histone H4 in amphibian mature oocytes are the first report that attempts to correlate the pattern of high acetylated H4 immunostaining with nuclear architecture in these specialized cell types.

Whereas a homogeneous H4 hyperacetylated reaction in the nucleus of the vitellogenic oocyte, a rather massive concentration of the hyperacetylated H4 was noticed towards the nucleus periphery. The occurrence of a H4 hyperacetylated homogeneous signal in the vitellogenic oocyte nucleus can be explained as a requirement of this chemical modification for the extensive transcription revealed in the nucleus of this type of oocytes. Many 'housekeeping' genes that are involved in establishing embryo segments and ribosomal genes are abundantly transcribed and even amplified in oogenesis.

The occurrence of the concentrated H4 hyperacetylated signal in the form of blocks at the periphery of the nucleus may be interpreted as follows: firstly as a reflection of the level of rDNA axes of extrachromosomal nucleoli involved in intense rRNA transcription, H4 hyperacetylated histone is accumulated; or secondly, as ribonucleoproteins that detach from extrachromosomal nucleoli are exported from the nucleus to the cytoplasm, H4 hyperacetylated histone remains at the periphery of the nucleus in order to be recycled and/or degraded. A peculiar aspect of the immunostaining pattern was the lack of signal for hacH4 in extrachromosomal nucleoli and in perinucleolar regions.

Other experiments performed on plants have shown that perinucleolar regions and nucleoli during S phase generally do not contain hyperacetylated forms of H4 (Jasencakova *et al.*, 2000) and are transcriptionally inactive (Shaw *et al.*, 1995). In vitellogenic oocytes, rDNA axes of extrachromosomal nucleoli are involved in an intense transcription activity, thus we interpreted these findings because of abundant deposition of ribonucleoproteins particles (RNP) at the nucleolar organizing region, covering the rDNA axes.

Chromatin immunoprecipitation experiments have shown that histone H4 is hyperacetylated in the promoter regions of active genes (Shestakova *et al.*, 2001; Zhao *et al.*, 2005; Workman, 2006). Recently, it has been demonstrated that distinct histone modification patterns function as recognition codes for the recruitment of different transcription factors upon transcriptional activation (Agalioti *et al.*, 2002). Thus the histone code may function as an epigenetic marker that is directly associated with transcriptional activation (Jenuwein & Allis, 2001).

Our RE-ChIP experiments performed for the first time on chromatin from previtellogenic and vitellogenic oocytes revealed that histone H4 at the level of rRNA genes promoter remains in a highly acetylated state during oocyte differentiation. However, significant dynamics of other chromatin epigenetic changes, such as H2A ubiquitinylation and cytosine methylation, are occurring concomitantly.

We noticed multiple epigenetic modifications at the rRNA gene promoter regions in previtellogenic oocytes. Thus, at the same copy level of ribosomal cistron promoter we found, using two RE-ChIP experiments, the presence of highly acetylated histone H4 and 5MeC, respectively; highly acetylated H4; and H2A ubiquitinylated. In this case it is very difficult to make any correlation with the transcriptional state of that gene. In previtellogenic oocytes, ribosomal genes are actively transcribed, but this activity has not been extensively observed in vitellogenic oocytes. Before yolk genesis, at the beginning of oocytes development, these genes may be marked for some important and intensive functional state.

It is possible that our experiment corresponded to the moment of oogenesis, which is needed to mark ribosomal genes for future transcriptional

activity. These hypotheses need further investigation, including the study of other components of the concerted chromatin conformation dynamics. As we had previously shown, some researchers have demonstrated that H4 is hyperacetylated in the promoter of active genes (Kuo *et al.*, 1998; Shestakova *et al.*, 2001).

Ubiquitinylation is a chemical modification involved in protein degradation via a proteasome pathway. Nevertheless, ubiquitinylation is not limited to degraded proteins, some studies have shown that this modification is also involved in rDNA repair, cell cycle control, cellular response to stress and histone code (Baarends *et al.*, 1999).

Immunohistochemical studies of meiotic male mouse cells evidenced that the amount of H2A-ubiquitinylated (uH2A) reaches its maximum during the pachytene stage of meiotic prophase. In early pachytene, uH2A is concentrated in the condensed and transcriptional inactive XY chromatin of the sex body. By mid-pachytene, uH2A is detected throughout the whole nucleus, but is subsequently limited to the sex body of late pachytene spermatocytes (Baarends *et al.*, 1999). At present, the function of histone ubiquitinylation is not clear. There are some indications that this histone modification results in destabilization of the H2A–H2B dimer, which may facilitate dimer displacement and DNA transcription (Li *et al.*, 1993). Our results support the hypothesis that uH2A may be a signal for H2A–H2B dimer displacement at the nucleosomal level. It might be possible that uH2A and hyperacetylated H4 at the level of our sequence of interest (544 bp) represent modifications with marking roles for the future functional state of the promoter.

Much work had been performed on the silenced gene promoter, which has revealed strong evidence for the presence of 5′-methyl cytosine at CpG dinucleotides (Bird, 1996, 2002; Nan *et al.*, 1998; Bird & Wolffe, 1999; Lawrence *et al.*, 2004). Methylation of cytosine bases located 5′ to a guanosine in a CpG dinucleotide is an epigenetic marker known to be involved in gene transcriptional repression (Jones & Baylin, 2002). Yet, until recently, the role of cytosine methylation in rRNA gene regulation was unclear (Grummt & Pikaard, 2002). Methylated ribosomal genes are transcribed efficiently when injected into oocyte nuclei or when transcribed *in vitro* (Macleod & Bird, 1983; Pennock & Reeder, 1984), suggesting that DNA methylation is not sufficient for transcriptional repression.

Our intriguing result that indicated a hyper-acetylated and, in the same time, a methylated promoter, might be explained by the fact that the ribosomal cistron could be inactive at our moment of investigation, but it might start the preparation for its future intense transcriptional activity. H4 hyperacetylation of the ribosomal gene promotor might be required to keep chromatin in a potentially relaxed or accessible state in which transcription can be induced as required.

It is obvious that the described chromatin dynamic changes could be ascribed to a specific complex epigenetic code for gamete differentiation. Consequently, chromatin may adopt an ‘open’ or a ‘closed’ conformation depending upon the functional state of that domain.

The lack of 5′-methyl cytosine and ubiquityl–H2A at the rRNA gene promoter region in vitellogenic oocytes allows us to conclude that, during oogenesis, a dynamic of epigenetic modification takes place. Nucleosomal core histones are targets for many post-translational modifications. All these epigenetic modifications act in a synergistic way to modulate DNA-binding protein accessibility, and might represent signals for the recruitment of transcription complexes.

Whereas the functional correlations between histone variants/histone modifications/DNA methylation appear to become increasing clear, in contrast the structural modifications of chromatin remain to be defined in most instances. An interesting possibility that has increasing experimental support is that some variants and modifications might operate synergistically. Moreover, it is possible that most histone variants and modifications operate *in vivo* in a concerted way, affecting either an individual histone or different histones.

Previously, the role of a single epigenetic modification was studied (Kaplan *et al.*, 1984, Ausio & van Holde, 1986, Garcia-Ramirez *et al.*, 1995). Taken together, the results of our present study have revealed that a dynamic epigenetic network mediates the transcriptional state of rDNA, by interplay of histone post-translational modification, linker histone variants, DNA methylation and other chromatin ATP-dependent remodelling complexes, in order to establish the complex functional state of rRNA genes.

References

Agalioti, T., Chen, G. & Thanos, D. (2002). Deciphering the transcriptional histone acetylation code for a human gene. *Cell* **111**, 381–92.

Allfrey, V., Faulkner, R.M. & Mirsky, A.E. (1964). Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. Natl. Acad. Sci. USA* **51**, 786–94.

Ausio, J. (1992). Structure and dynamics of transcriptionally active chromatin *J. Cell. Sci.* **102**, 1–5.

Ausio, J. & van Holde, K.E. (1986). Histone hyperacetylation: its effect on nucleosome conformation and stability. *Biochemistry* **25**, 1421–8.

Ayer, D.E. (1999). Histone deacetylases, transcriptional repression with SINers and NuRDs. *Trends Cell Biol.* **9**, 193–8.

https://doi.org/10.1017/S0967199408004863 Published online by Cambridge University Press

Baarends, W.M., Hoogerbrugge, J.W., Roest, H.P., Jan Vreeburg, M.O., Hoeijnakers, H.J. & Grootegoed, J.A. (1999). Histone ubiquitination and chromatin remodelling in mouse spermatogenesis. *Dev. Biol.* **207**, 322–33.

Barsacchi, G., Bussoti, L. & Mancino, G. (1970). The maps of the lampbrush chromosomes of *Triturus* (Amphibia, Urodela). IV *Triturus vulgaris meridionalis*. *Chromosoma* **31**, 255–78.

Bird, A.P. (1996). The relationship of DNA methylation to cancer. *Cancer Surv.* **28**, 87–101.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev.* **16**, 6–21.

Bird, A. & Wolffe, A.P. (1999). Methylation-induced repression—belts, braces and chromatin. *Cell* **99**, 451–4.

Brown, D. & Dawid, J.B. (1970). Specific gene amplification in oocyte. *Science* **160**, 272.

Brown, D.T., Gunjan, A., Alexander, B.T. & Sittman, D.B. (1997). Differential effect of H1 variant overproduction on gene expression is due to differences in the central globular domain. *Nucl. Acids Res.* **15**, 5003–9.

Burlibasa, L. & Gavrila, L. (2005). Molecular and ultrastructural studies of the sperm chromatin from *Triturus cristatus*. *Zygote* **13**, 197–205.

Carruthers, L.M., Bednary, J., Woodcock, C.L. & Hansen, J.C. (1998). Linker histones stabilize the intrinsic salt-dependent folding of nucleosomal arrays, mechanistic ramifications for higher-order chromatin folding. *Biochemistry* **37**, 14776–87.

Conconi, A., Widmer, R.M., Koller, T. & Sojo, Y.M. (1989). Two different chromatin structure coexist in ribosomal RNA genes throughout the cell cycle. *Cell* **57**, 753–61.

Garcia-Ramirez, M., Rocchini, C. & Ausio, J. (1995). Modulation of chromatin folding by histone acetylation. *J. Biol. Chem.* **270**, 17920–8.

Grant, A.P. (2001). A tale of histone modifications. *Genome Biol.* **2**(4), 0003.1–6.

Grummt, I. & Pikaard, C.S. (2003). Epigenetic silencing of RNA polymerase I transcription. *Nat. Rev. Mol. Cell. Biol.* **4**, 641–9.

Grunstein, M. (1990). Histone function in transcription. *Ann. Rev. Cell. Biol.* **6**, 643–78.

Gunjan, A. & Brown, D.T. (1999). Overproduction of histone H1 variants *in vivo* increases basal and induced activity of the mouse mammary tumor virus promoter. *Nucleic Acids Res.* **27**, 3355–63.

Gunjan, A., Sittman, D. & Brown, D. (2001). Core histone acetylation is regulated by linker histone stoichiometry *in vivo*. *J. Biol. Chem.* **276**(5), 3635–40.

Jaenish, R. & Bird, A. (2003). Epigenetic regulation of gene expression, how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33**, 245–54.

Jasencakova, Z., Meister, A., Walter, J., Turner, B.M. & Schubert, I. (2000). Histone H4 acetylation of euchromatin and heterochromatin is cell cycle dependent and correlated with replication rather than with transcription. *Plant Cell* **12**, 2087–100.

Jenuwein, T. & Allis, C D. (2001). Translating the histone code. *Science* **293**, 1074–80.

Jones, P.A. & Baylin, S.B. (2002). The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* **3**, 415–28.

Juan, L.J., Utley, R.T., Adams, C.C., Vetesse-Dadey, M. & Workman, J.L. (1994). Differential repression of transcription factor binding by histone H1 is regulated by the core histone amino termini. *EMBO J.* **13**, 6031–40.

Kandolf, H. (1994). The H1A histone variant is an in vivo repressor of oocyte-type 5S gene transcription in *Xenopus laevis* embryos. *Proc. Natl. Acad. Sci. USA* **91**, 7257–61.

Kaplan, L.J., Bauer, R., Morrison, E., Langan, T.A. & Fosman, G.D. (1984). The structure of chromatin reconstituted with phosphorylated H1. Circular dichroism and thermal denaturation studies. *J. Biol. Chem.* **259**, 8777–85.

Karpen, G.H., Schaefer, J.E. & Laird, C.D. (1998). A *Drosophila* rRNA gene located in euchromatin is active in transcription and nucleolus formation. *Genes Dev.* **2**, 1745–63.

Kim, J.M., Liu, H., Tazaki, M., Nagata, M. & Aoki, F. (2003). Changes in histone acetylation during mouse oocyte meiosis. *J. Cell Biol.* **162**(1), 37–46.

Kuo, M.H., Zhou, J., Jambeck, P., Churchill, M.E. & Allis, C.D. (1998). Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo. *Genes Dev.* **12**, 627–39.

Lawrence, R.J., Early, K., Pontes, O., Silva, M., Chen, Z.J., Neves, N., Viegas, W. & Pikaard, C.S. (2004). A concerted DNA methylation/histone methylation switch regulates rRNA gene dosage control and nucleolar dominance. *Mol. Cells* **13**, 599–609.

Long, E.O. & Dawid, I.B. (1980). Repeated genes in eukaryotes. *Ann. Rev. Biochem.* **4**, 727–64.

Lusser, A. (2002). Acetylated, methylated, remodelled, chromatin states for gene regulation. *Curr. Opin. Plant. Biol.* **5**, 437–43.

Macgregor, H.C. (1993). *An Introduction to Animal Cytogenetics*. London: Chapman & Hall.

Macleod, D. & Bird, A. (1983). Transcription in oocytes of highly methylated rDNA from *Xenopus laevis* sperm. *Nature* **306**, 200–3.

Magnaghi-Jaulin, L. & Jaulin, C. (2006). Histone deacetylase activity is necessary for chromosome condensation during meiotic maturation in *Xenopus laevis*. *Chromosome Res.* **14**, 319–32.

McStay, B. (2006). Nucleolar dominance, a model for rRNA gene silencing. *Genes Dev.* **20**, 1207–14.

Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N. & Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**, 386–9.

Peng, J.C. & Karpen, G.H. (2007). H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. *Nat. Cell. Biol.* **9**(1), 25–35.

Pennock, D.G. & Reeder, R.H. (1984). In vitro methylation of *Hpa*II sites in *Xenopus laevis* rDNA does not affect its transcription in oocytes. *Nucleic Acids Res.* **12**, 2225–32.

Polach, K.J. & Widom, J. (1999). Restriction enzymes as probes of nucleosome stability, *Methods Enzymol.* **304**, 278–98.

Reeder, R.H. (1985). Mechanisms of nucleolar dominance in animals and plants. *J. Cell Biol.* **101**, 2013–6.

Santoro, R. (2005). The silence of the ribosomal RNA genes. *Cell Mol. Life Sci.* **62**, 2067–79.

Santoro, R. & Grummt, I. (2005). Epigenetic mechanism of rRNA gene silencing, temporal order of NoRC-mediated

histone modification, chromatin remodelling and DNA methylation. *Mol. Cell. Biol.* **25**, 2539–46.
Santoro, R., Li, J. & Grummt, I. (2002). The nucleolar remodelling complex NoRC mediates heterochromatin formation and silencing of ribosomal gene transcription. *Nat. Gen.* **32**, 393–6.
Shaw, P.J., Highett, M.I., Beven, A.F. & Jordan, E.G. (1995). The nucleolar architecture of polymerase I transcription and processing. *EMBO J.* **14**, 2896–906.
Shen, W. & Gorovsky, M.A. (1996). Linker histone H1 regulates specific gene expression but not global transcription in vivo. *Cell* **86**(3), 475–83.
Shertakova, E., Bandu, M.T., Doly, J. & Bonnefoy, E. (2001). Inhibition of histone induces constitutive depression of the b interferon promoter and confers antiviral activity. *J. Virol.* **75**, 3444–52.
Sommerville, J., Baird, J. & Turner, B. (1993). Histone H4 acetylation and transcription in amphibian chromatin. *J. Cell Biol*. **120**(2), 277–90.
Steopoe, I., Gavrilă, L., Mihăescu, Gr & Comşa, D. (1985). The study of the lampbrush chromosomes in relation with the ribosomal gene amplification in oocytes of Triturus cristatus and Salamandra salamandra. *Annals of Buch. Univ.* **XXXIV**, 3–9.
Strahl, B.D. & Allis, C.D. (2000). The language of covalent histone modifications. *Nature* **403**, 41–5.
Turner, B.M. (1991). Histone acetylation and control of gene expression. *J. Cell. Sci*. **99**, 13–20.
Turner, B.M. (1993). Decoding the nucleosome. *Cell* **75**, 5–8.
Ura, K., Hayes, J.J. & Wolffe, A.P. (1995). A positive role for nucleosome mobility in the transcriptional activity of chromatin templates, restriction by linker histones. *EMBO J.* **14**, 3752–65.
Utley, R.T., Ikeda, K., Grant, P.A., Cote, J., Steger, D.J., Eberharter, A., John, S. & Workman, J.L. (1998). Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. *Nature* **394**, 498–502.
Wang, L., Liu, L. & Berger, S.L. (1998). Critical residues for histone acetylation by Gcn5, functioning in Ada and SAGA complexes, are also required for transcriptional function *in vivo*. *Genes Dev*. **12**, 640–53.
Wolffe, A.P. (1992). *Chromatin, Structure and Function*. London: Academic Press.
Workman, J.L. (2006). *Nucleosome displacement in transcription*, *Genes Dev*. **20**, 2009–17.
Zărnescu, O. (2007). Immunohistochemical distribution of hyperacetylated histone H4 in testis of paddlefish *Polyodon spathula*, ultrastructural correlation with chromatin condensation. *Cell Tissue Res*. DOI 10.1007/s00441-006-0373-3.
Zhao, J., Herrera-Diaz, J. & Gross, D.S. (2005). Domain-wide displacement of histones by activated heat shock factor occurs independently of Swi/Snf and is not correlated with RNA polymerase II density. *Mol. Cell. Biol*. **25**, 8985–99.
Zlatanova, J. (1990). Histone H1 and regulation of transcription of eukaryotic genes. *Trends Biochem. Sci*. **15**, 2.