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# Polymorphic Heterochromatic Segments in Japanese Quail Microchromosomes

A. Krasikova A. Daks A. Zlotina E. Gaginskaya

Saint-Petersburg State University, Saint-Petersburg, Russia

**Key Words**
Avian microchromosomes · Centromere · Heterochromatin · Histone modifications · Lampbrush chromosomes

**Abstract**
Using highly extended lampbrush chromosomes from diplotene oocytes, we have examined the distribution of heterochromatin protein 1 β (HP1β) and histone H3 modifications on chicken *(Gallus gallus)* and Japanese quail *(Coturnix japonica)* (2n = 78) microchromosomes. Acrocentric microchromosomes of chicken and submetacentric microchromosomes of quail differ in several morphological features. In addition to pericentromeric and subtelomeric blocks of constitutive heterochromatin, which are enriched in HP1β protein and repressive histone modifications, not completely condensed but heterochromatic segments were found to be an attribute of the short arms of submetacentric microchromosomes in Japanese quail. These heterochromatic regions are variable in length and do not form chiasmata in female germ cells. Dissimilarity in the centromere positions in chicken and Japanese quail microchromosomes is proposed to be due to the accumulation of repetitive sequences on the short arms of quail microchromosomes. Transcriptional activation of polymorphic heterochromatic segments of quail microchromosomes during the lampbrush stage is demonstrated.



Karyotypes of domestic birds are very complicated for cytogenetic analysis [Schmid et al., 2005; Griffin et al., 2007]. In chicken *(Gallus gallus)* and Japanese quail *(Coturnix japonica)* (2n = 78), only 10 pairs of chromosomes, including 9 pairs of the largest autosomes and sex chromosomes, are cytologically distinguishable, while the rest of the chromosomes called 'microchromosomes' are difficult to identify. The small size of avian microchromosomes constrains the construction of detailed cytological maps, precise physical gene mapping and analysis of intrachromosomal rearrangements accompanying karyotype evolution. At the same time, avian microchromosomes are gene-rich, have all characteristics typical for mammalian T-bands (GC-richest subfraction of R-bands) and may represent ancestral syntenies conserved for over 400 million years [Rodionov, 1996; Schmid et al., 2000; Andreozzi et al., 2001; Burt, 2002].

Due to the giant size, characteristic chromomere-loop appearance and enrichment with cytological landmarks, usage of diplotene chromosomes in their lampbrush condition significantly increases the resolution of cytogenetic analysis [Solovei et al., 1998; Galkina et al., 2006; Krasikova et al., 2006; Deryusheva et al., 2007; Penrad-Mobayed et al., 2009]. Indeed, during the lampbrush stage of oogenesis the length of avian chromosomes increases more than 30-fold. Detailed description and cytological maps of chicken and quail lampbrush macrochromosomes are available [Chelysheva et al., 1990; Rodionov

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Alla Krasikova
Saint-Petersburg State University
Oranienbaumskoie sch. 2, Stary Peterhof
Saint-Petersburg 198504 (Russia)
Tel. +7 812 450 7311, Fax +7 812 450 7310, E-Mail spbchromas@gmail.com

and Chechik, 2002; Derjusheva et al., 2003, Schmid et al., 2005], whereas lampbrush microbivalents from oocytes of these species are less characterized. The present investigation is devoted to the comparative analysis of chicken and Japanese quail lampbrush microchromosomes.

Previously, the majority of chicken microchromosomes were found to be acrocentric [Solari, 1980; Kaelbling and Fechheimer, 1983; Krasikova et al., 2006]. In contrast, 2 independent high-resolution studies using either pachytene or lampbrush karyotypes have demonstrated that most microchromosomes in Japanese quail are submetacentric [Calderon and Pigozzi, 2006; Krasikova et al., 2006]. Dissimilarity in the centromere positions in chicken and quail microchromosomes was verified by FISH with pericentromeric CNM and *Bgl*II repeats applied to lampbrush chromosomes (LBCs) [Krasikova et al., 2006]. Furthermore, Calderon and Pigozzi [2006] emphasized that among 17 examined species of birds Japanese quail was the only species with a large number of biarmed microchromosomes. At the same time, an extremely low rate of inter- and intrachromosomal rearrangements in chicken and Japanese quail have been continually reported [Schmid et al., 2000, 2005; Shibusawa et al., 2001, 2004; Guttenbach et al., 2003; Galkina et al., 2006; Kayang et al., 2006; Sasazaki et al., 2006]. Particularly, no data supporting the existence of interchromosomal rearrangements among microchromosomes was presented. A number of clones containing genomic sequences from single chicken microchromosomes hybridized to microchromosomes of similar size in quail [Shibusawa et al., 2001; Kayang et al., 2006]. Moreover, comparative studies between chicken and turkey have provided further evidence for conservation of microchromosomes in Galliformes [Griffin et al., 2008].

Because of the high conservation of Galliformes chromosomes, the difference in centromere positions between chicken and Japanese quail microchromosomes could be explained by (1) multiple pericentric inversions, (2) formation of centromeres de novo and (3) accumulation of heterochromatin on the quail microchromosomes. Since centromere indexes differ in the majority of microchromosomes of these 2 closely related species, we suggest that the latter opportunity is more likely. Thus the primary task of this study was to describe and compare various types of heterochromatin within chicken and Japanese quail microchromosomes. With this object in mind we investigated the distribution of heterochromatin protein 1 (HP1) and histone H3 modifications within different segments of lampbrush microbivalents of these species.

## Materials and Methods

Chicken *(G. g. domesticus)* and Japanese quail *(C. japonica)* LBCs were isolated manually from oocytes of 1.0–1.5 mm in diameter according to the standard technique [Solovei et al., 1994]. About 40 oocytes per animal were used for preparation of LBC spreads. Adult females (20 animals) of the same population were bought from commercial stocks. Immunostaining of chicken and quail LBC was carried out as previously described [Krasikova et al., 2005] with the following rabbit polyclonal antibodies: K828 against STAG2, a centromere marker on LBCs, ab8580 against H3K4me3 (Abcam), ab71999 against H3K9me3 (Abcam), 07-449 against H3K27me3 (Upstate), as well as mouse monoclonal antibodies: antibody directed against HP1β (Eurogentec), H14 (BAbCO) and V22 (kindly provided by U. Scheer and R. Hock) against the phosphorylated C-terminal domain of RNA polymerase II, and sc-32724 against the trimethylguanosine (TMG) cap of most of the splicing snRNAs (Santa Cruz). After immunostaining and image acquisition, some LBC preparations were used for FISH. LBCs were hybridized with the following fluorochrome-labeled oligonucleotides [Deryusheva et al., 2007]: CNMneg (Cy5), 5′-AAATGGGGGATTTTCGAAGAGAAAACA-3′; CCOneg (Cy5), 5′-ACATCTGCCCCACAGCAGCTCCTGCCCCAT-3′.

Oligonucleotides were designed according to the consensus sequences of 41-bp CNM [Matzke et al., 1990] and *Bgl*II [Tanaka et al., 2000] repeats. The whole-chromosome paints F13 and F15, specific for the individual chicken chromosomes (GGA) 6 and 7 [Griffin et al., 1999], were also used. FISH was performed according to DNA/(DNA+RNA) hybridization protocol [Krasikova et al., 2006]. Preparations were mounted in antifade solution with DAPI or chromomycin A3 fluorochromes and then examined using a Leica fluorescence microscope DM4000 equipped with a monochrome digital camera DFC350 FX and appropriate filter cubes.

3D-preserved nuclei, microsurgically isolated from quail oocytes, were stained with nucleic acid-specific fluorochrome Sytox green (Invitrogen) and analyzed by confocal laser scanning microscopy with a Leica TCS SP5 microscope equipped with an argon (496 nm) laser (Leica Mikrosysteme, Bensheim, Germany). For computational analysis of 3D image stacks and 3D reconstructions Las AF (Leica) software was used.

## Results and Discussion

### *Morphology of Lampbrush Microbivalents*

In the lampbrush form every chicken and quail microbivalent usually has 1 chiasma and a chromosome-specific pattern of chromomeres [Rodionov et al., 1992; Rodionov and Chechik, 2002]. Previously we have shown that centromeric regions of avian LBCs are marked by cohesin-enriched structures [Krasikova et al., 2006]. Precise centromere positioning in the complete sets of chicken and quail LBCs using immunofluorescent staining with anti-cohesin antibodies allowed us to extend our previous data. In all chicken microchromosomes ex-

cept GGA10, the centromere localizes between 2 bright chromomeres at 1 of the chromosome ends (fig. 1a). In contrast, the centromeres on the majority of quail lampbrush microchromosomes do not localize adjacent to the prominent subtelomeric chromomeres (fig. 1a). In the quail karyotype among 29 pairs of microchromosomes, only 3 pairs of chromosomes could be univocally classified as acrocentric. The following scheme illustrates the difference in chromosome morphology between chicken acrocentric and quail submetacentric lampbrush microchromosomes (fig. 1f). In chicken microchromosomes, DAPI-positive chromomeres flank putative centromeres, whereas in quail biarmed microchromosomes, pericentromeric chromomeres are usually chromomycin-positive (data not shown). Our observations indicate that microchromosomes in chicken and quail differ in their length, quail microchromosomes being somewhat longer (fig. 1a–d). At this point one important detail must be noted: in many submetacentric microchromosomes of quail a chromatin domain between the tightly condensed pericentromeric chromomeres and the terminal chromomere of the short arm is represented by an array of compact chromomeres with relatively small lateral loops (fig. 1a, asterisks). Chromosome regions with similar morphology are absent in acrocentric lampbrush microchromosomes of both chicken and Japanese quail.

*Polymorphic Segments in Quail Microchromosomes*

In some instances in the chromosome complements from quail oocytes there may be bivalents in which homologous chromosomes differ in the length of their short arms. Microbivalents in figure 1 (right panel) can serve as a cytological illustration of such polymorphism. Analysis of intact nuclei isolated from quail oocytes by confocal laser scanning microscopy demonstrated that asymmetric microbivalents can be clearly distinguished in 3D reconstructions (fig. 1e) and thus are not a result of chromosome microsurgical isolation. Appearance of asymmetric microbivalents could be also explained by chiasma formation between non-homologous microchromosomes. However this is unlikely, since chromomere-loop pattern in the longer arms is usually similar in the 2 chromosomes which form asymmetric microbivalents. The overall number of asymmetric microbivalents in lampbrush karyotypes can vary between individuals; in several cases 3 dissimilar homologous pairs in full sets of quail LBCs were recorded. Using immunofluorescent staining we determined that in these bivalents the longer homologue is submetacentric, while the shorter chromosome is often acrocentric (fig. 1a, right panel). This leads to certain difficulties in determining the precise number of acrocentric chromosomes in the quail karyotype, which can be either homozygous for microchromosomes with prominent short arms or homozygous for microchromosomes without these regions. To test whether certain quail microchromosomes comprise polymorphic heterochromatic segments, a detailed comparative analysis of distribution of inactive chromatin markers in chicken and quail LBCs has been made.

*Distribution of Heterochromatin Protein 1 and Histone H3 Modifications*

In order to examine the distribution of HP1β, which is known to be involved in formation and maintenance of heterochromatin and sister-chromatid cohesion [Hiragami and Festenstein, 2005], we performed immunofluorescence staining of LBC spreads. Within chicken and quail oocyte karyotypes, the most intensive signals were observed in chromomeres of the W chromosome (data not shown), which consists mostly of tandem repeats. Bright labeling was also found in chromomeres at pericentromeric and subtelomeric regions of all lampbrush bivalents including the smallest ones (fig. 1b). The distribution of HP1β in the quail submetacentric lampbrush microchromosomes deserves special attention. In 1 of the 2 arms of these microchromosomes, the chromatin between pericentromeric and terminal chromomeres was also significantly enriched with the HP1β protein (fig. 1b, central panel). In other regions, all chromomeres are stained with the intensity that is proportional to the amount of DNA.

Staining of isolated LBCs with antibodies against histone modifications associated with inactive chromatin allowed us to reveal additional differences between chicken and quail microchromosomes. Trimethylation of histone H3 at lysine 27 (H3K27me3) correlates with gene repression and can be found in pericentromeric and other heterochromatic regions of chromosomes [Peterson and Laniel, 2004]. In chicken and quail LBC spreads, antibody against H3K27me3 produced mainly chromomere staining on all chromosomes, the pattern being similar in both homologues. Preferential staining of pericentromeric chromomeres of LBCs is demonstrated. In the majority of quail microchromosomes, the inner region of the short arm displays a higher level of histone H3K27me3 than the inner region of the longer arm (fig. 1d).

Histone H3 trimethylated at lysine 9 (H3K9me3) is associated with transcriptional silencing and is typical of regions of constitutive heterochromatin [Peterson and

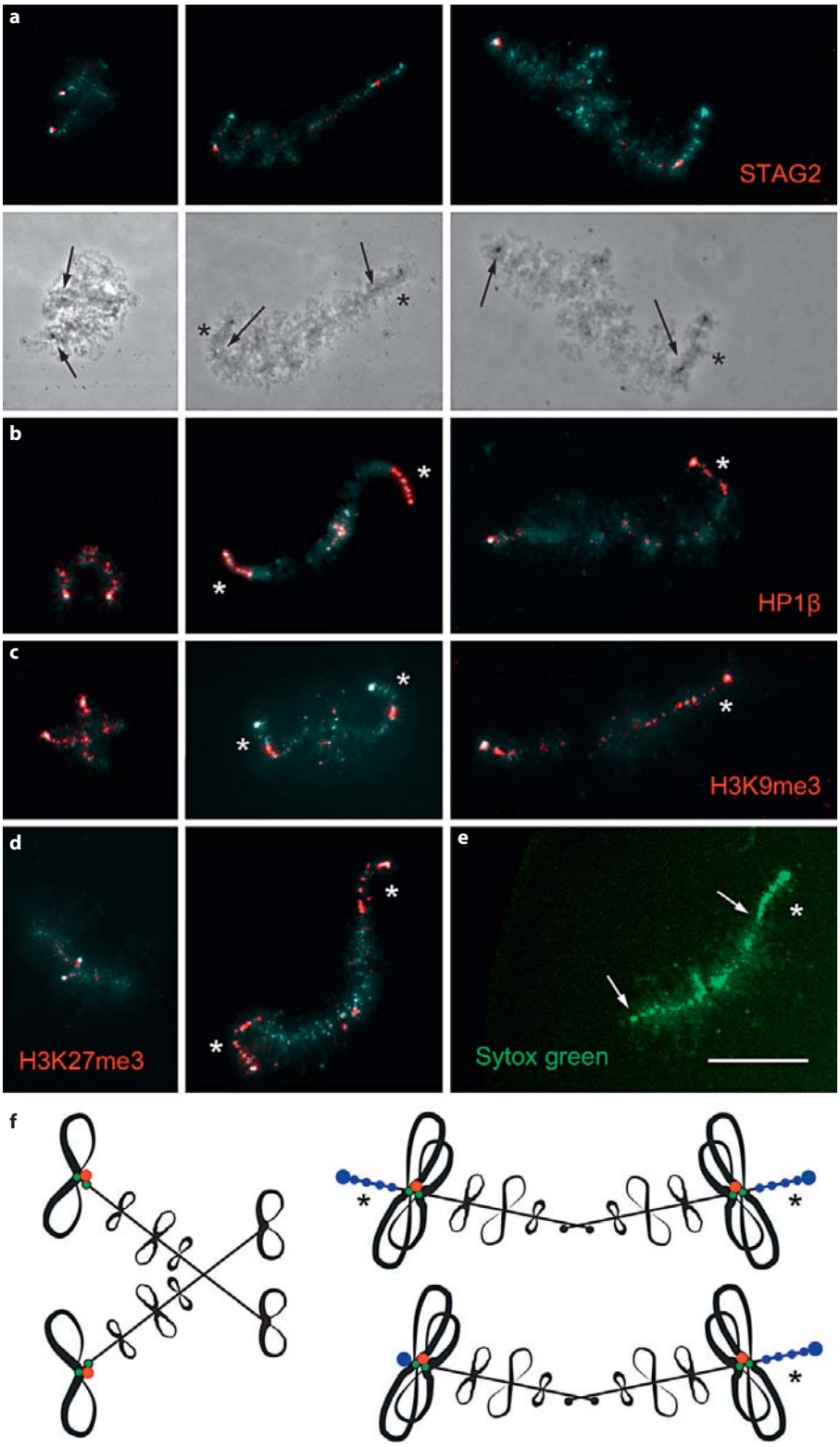


**Fig. 1. a–d** High-resolution comparative analysis of the distribution of heterochromatin markers on chicken acrocentric (left panel) and Japanese quail submetacentric (central panel) lampbrush microchromosomes. Asymmetric microbivalents from quail oocytes are shown on the right panel. **a** Immunostaining with an antibody against STAG2 (red signal), showing the positions of centromeres (arrows). Corresponding phase-contrast images are shown. **b** Immunodetection of HP1β protein (red signal) in the heterochromatic regions of chromosomes. **c**, **d** Immunofluorescent staining with antibodies against H3K9me3 (**c**) and H3K27me3 (**d**) (red signal). Asterisks indicate the short arms of quail microchromosomes. Chromosomes are counterstained with DAPI. Scale bar = 10 μm. **e** Microbivalent with polymorphism present on the short arm within the intact oocyte nucleus of Japanese quail. **f** Schematic drawings of representative chicken (left) and quail (right) lampbrush microbivalents, in which 2 homologues are united by chiasma. Centromeres (red), pericentromeric chromomeres (green) and heterochromatic arms (blue) are indicated. In quail asymmetric microbivalents, the longer homologue is submetacentric, while the shorter one is acrocentric.

Laniel, 2004]. In avian lampbrush microchromosomes, higher intensity of labeling with antibody against H3K9me3 was observed in the pericentromeric and terminal chromomeres (fig. 1c). Other chromomeres of LBCs were moderately stained. Therefore chromomeres in the centromeric and subtelomeric regions of lampbrush microchromosomes are predominantly enriched with H3K9me3.

The positions of heterochromatic regions were defined by enrichment with HP1β and histone modifications typical for silent chromatin and were compared with the locations of pericentromeric arrays of CNM and *Bgl*II repeats in chicken and quail respectively. It can be concluded, that in chicken microchromosomes, there are prominent subtelomeric and pericentromeric blocks of heterochromatin, which generally remain condensed during the lampbrush stage. In quail submetacentric microchromosomes, in addition to subtelomeric and pericentromeric chromomeres, chromomeres of the short arm were found to demonstrate significant enrichment with HP1β and H3K27me3, a modification associated with heterochromatin. It is worthy noting that the heterochromatic short arms of the biarmed micro-LBCs in quail, however, do not display high levels of condensation which is typical for pericentromeric or terminal chromomeres (fig. 1, asterisks).

As it was expected, in asymmetric quail microbivalents the amount of heterochromatic material is different in 2 homologues, leading to variation in centromere position. After immunostaining of such bivalents with antibodies against either HP1β or H3K27me3, the short arm of the longer submetacentric homologue was brightly labeled while acrocentric homologues did not bear such blocks (fig. 1b). Therefore, in the quail karyotype, the absence of the block which is enriched with heterochromatin markers usually results in a change of microchromosome morphology from submetacentric to acrocentric (fig. 1f).

To further characterize quail microchromosomes in the lampbrush form, we checked them for the presence of active chromatin marks. One of the histone modifications that is associated with transcriptionally active chromatin is histone H3 trimethylated at lysine 4 (H3K4me3) [Peterson and Laniel, 2004]. The non-repressive H3K4me3 modification was detected in the chromatin of laterally projecting loops which arise from chromomeres in both arms of quail microchromosomes, although loops of the p arms were labeled less intensively (fig. 2a). Likewise, in chicken lampbrush microchromosomes, staining with H3K4me3 antibody shows a punctuated pattern on the lateral loops of the longer arms. Importantly, this modification of histone H3 is not excluded from chromomeres (fig. 2a).

In addition, the antibody against the hyperphosphorylated C-terminal domain of RNA polymerase II revealed uniform staining along the DNP axes of lampbrush lateral loops including polymorphic regions of biarmed microchromosomes (fig. 2c), which are characteristic of Japanese quail. Organized into small loops, the transcription units in the short arms of quail micros can be visualized at the cytological level due to the existence of RNP matrix. Noticeably, RNP matrix of the lateral loops in variable regions contains splicing small nuclear RNAs equally to the lateral loops on the longer arms (fig. 2b). In contrast, splicing snRNAs are not detectable in the matrix of loops bearing transcripts of 41-bp repeats [Deryusheva et al., 2007]. These results allow us to conclude that the inner regions of the short arms of quail submetacentric microchromosomes are represented by blocks of non-silent heterochromatin which is not completely condensed.

*Chiasmata Distribution*

It is reasonable to mention here that all microbivalents in chicken and Japanese quail have at least 1 chiasma due to the exceptionally high meiotic recombination rate [Rodionov, 1996]. However, in quail females, we never observed chiasmata formation in the inner regions of heterochromatic arms of submetacentric diplotene microbivalents resulting in asymmetry in chiasmata distribution (fig. 1a–d, central panel; fig. 2). That leads to the suggestion that either crossovers are repressed in the polymorphic regions or hot spots of recombination are located in the other arm (usually the longer one) where 1 chiasma was constantly observed. It is in agreement with the notion that conventional crossing over is infrequent in the heterochromatin. Similarly, in *Triturus cristatus carnifex*, C-band-positive heteromorphic arms of chromosome I never form chiasmata and in the lampbrush form display presence of nascent satellite DNA transcripts [Morgan, 1978; Varley et al., 1980]. An intercalary accumulation of highly repeated sequences in the long arm of chromosome I of *T. c. carnifex* has been proposed. It is important to emphasize that in quail microbivalents heterozygous for heterochromatic segments, the only chiasma regularly forms in the distal euchromatic regions of the longer arms (fig. 1a–c, right panel; fig. 1e).

Another important characteristic of Japanese quail microchromosomes comes from a Zoo-FISH comparative study with painting probes from those chicken mac-

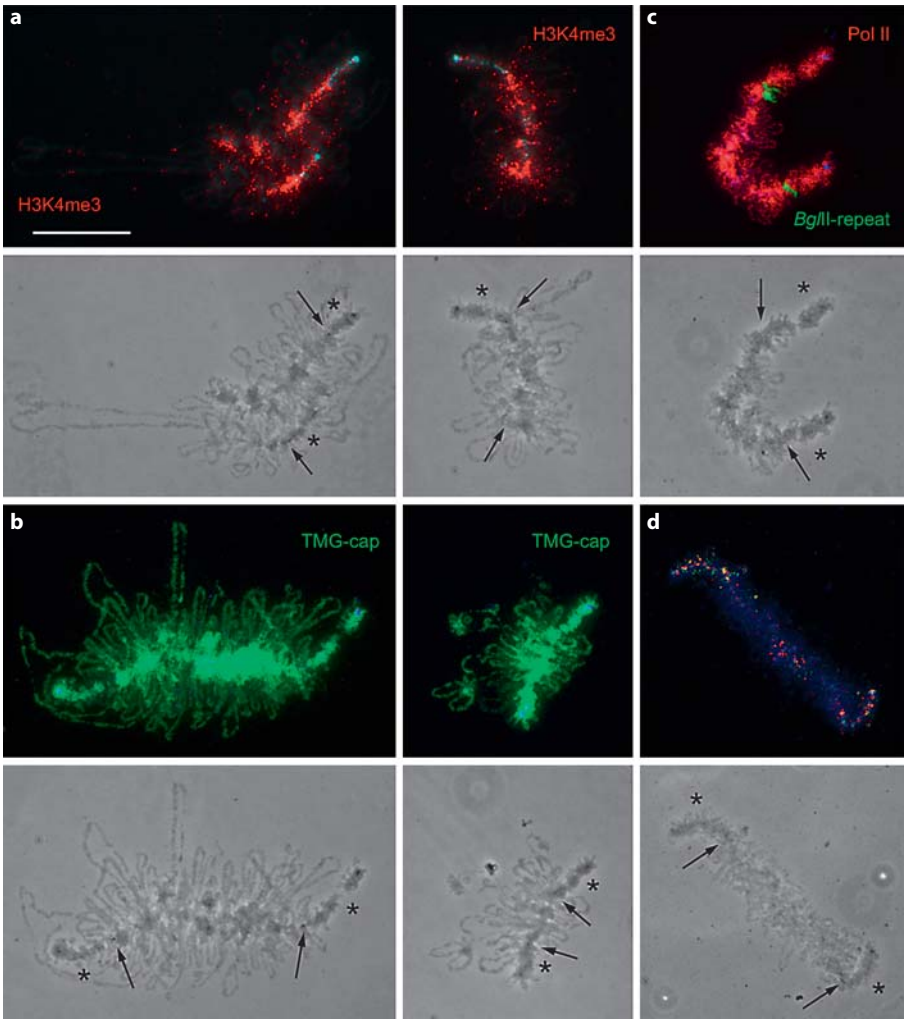


**Fig. 2.** Transcriptionally active chromatin in the polymorphic regions of quail submetacentric microchromosomes in the lampbrush form. **a**, **b** Immunofluorescent staining with antibodies against H3K4me3 (red signal) (**a**) and TMG cap of small nuclear RNAs (green signal) (**b**). **c** Immunodetection of hyperphosphorylated C-terminal domain of RNA polymerase II (red signal) in the lampbrush lateral loops followed by FISH with the *Bgl*II repeat (green signal). **d** Labeling of the short arms of quail submetacentric lampbrush microchromosomes with GGA6- (red signal) or GGA7- (green signal) painting probes. Corresponding phase contrast images are shown. Asterisks indicate the short arms of microchromosomes, arrows indicate the centromeres. Chromosomes are counterstained with DAPI. Scale bar = 10 μm.

rochromosomes that do not bear PO41 repeats, the latter being copious in chicken and Japanese quail genomes [Deryusheva et al., 2007]. In particular, in experiments where RNAase treatment was omitted and no Cot DNA was used as competitor, paints for GGA6 or GGA7 labeled not only their corresponding macrobivalents in quail LBC sets but also the short arm of submetacentric microchromosomes (fig. 2d). Whereas the same paints applied to quail metaphase chromosomes under standard Zoo-FISH conditions specifically hybridize to individual macrochromosomes [Guttenbach et al., 2003]. This indicates that polymorphic regions of quail microchromosomes contain unknown repetitive sequences complementary to a component of chicken macrochromosome-painting probes thus reinforcing our previous conclusions.

## Conclusion

Until now, the distribution of HP1β protein and histone H3 methylation marks in LBCs has not been characterized. In this study, H3K9me3, being an indicator of constitutive heterochromatin, was found to be significantly enriched in pericentromeric and subtelomeric chromomeres of avian LBCs. Interestingly, in the corresponding heterochromatic regions of *Xenopus* LBCs, in particular in terminal chromomeres, no incorporation of Flag-tagged histone H3 was detected, in contrast to the axis of lateral loops [Stewart et al., 2006]. Furthermore, in avian LBCs, H3K27me3 and HP1β localize in all lampbrush chromomeres, being especially abundant in the regions of constitutive heterochromatin. These results clearly demonstrate differences in chromatin modifications in lampbrush chromomeres that localize in the re-

gions of constitutive heterochromatin and all other chromomeres.

Quite the opposite, the non-repressive modification H3K4me3 is associated with the transcription units on laterally projecting loops of all avian LBCs where active RNA synthesis takes place. These observations are compatible with the data of Sommerville et al. [1993], who detected the association of histone H4 acetylation with the transcriptionally active chromatin on LBC lateral loops.

Detailed characterization of chicken and Japanese quail microchromosomes using highly extended LBCs as a model demonstrated that 1 of the 2 arms of quail submetacentric microchromosomes is enriched with HP1β and repressive histone modifications such as H3K27me3. However, these regions do not reveal the presence of PO41 and *Bgl*II repeats [Deryusheva et al., 2007], do not accumulate significant amounts of H3K9me3 and thus differ from pericentromeric and subtelomeric chromomeres. According to our data, differences in the positions of the centromeres between chicken and Japanese quail microchromosomes are probably due to the accumulation of the specific heterochromatin on the short arms of quail microchromosomes. At the same time we can not leave out the possibility of intrachromosomal rearrangements or centromere repositioning at least in some microchromosomes, both opportunities being also worthy to explain the difference in centromere positions. To check this, comparative BAC mapping on highly extended chicken and Japanese quail lampbrush microchromosomes should be performed.

It should be taken into account that despite a very small level of interspecific variation of avian genome size, the C-value of *G. g. domesticus* is estimated as 1.25 pg, while the C-value of *C. japonica* is estimated as 1.41 pg with intraspecific variation of 2.5% [Tiersch and Wachtel, 1991; animal genome size database: http://www.genomesize.com/]. In addition to the earlier documented cases of variation in quail karyotype, such as variation in length of the short arm of chromosome 4 and C-band at the terminal region of the short arm of the Z chromosome [de la Seña et al., 1991; Galkina et al., 2006], we demonstrate the occurrence of polymorphism on the presence of the short heterochromatic arms in microchromosomes. No phenotypic alterations or changes in oocyte morphology in individuals with heteromorphic microchromosome pairs were observed. Therefore, the inner region of the short arm of at least several submetacentric microchromosomes of quail seems to be dispensable. These regions strongly resemble subterminal domains of supernumerary B chromosomes in plants, which contain specific high copy repeats, are transcriptionally active and form inconsistent heterochromatin with unusual combinations of apparently conflicting chromatin modifications: trimethylated H3K4 and methylated H3K27 [Carchilan et al., 2007].

Our observations suggest that achiasmate HP1β-enriched arms of quail microchromosomes represent a specific type of heterochromatin which is transcriptionally active during the lampbrush stage of oocyte growth. Detailed analysis of the distribution of heterochromatin proteins in polythene chromosomes of *Drosophila* has demonstrated that HP1 could serve as a marker of β-heterochromatin [James et al., 1989], which is characterized by a low content of unique DNA sequences and enrichment with presumably 'dead' mobile genetic elements [Holmquist and Ashley, 2006]. We propose that repetitive sequences accumulate to form the major part of the variable arms of quail microchromosomes. Their transcription during a particular stage of oogenesis could be explained in terms of activation of interspersed retrotransposons which is apparently a typical feature of LBCs. We are currently investigating the nature of these heterochromatic regions and the distribution of mobile genetic elements along chicken and Japanese quail microchromosomes.


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