Chromosoma (2015) 124:341–351
DOI 10.1007/s00412-014-0502-9

RESEARCH ARTICLE

Epigenetic modifications in sex heterochromatin of vole rodents

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Received: 12 September 2014 / Revised: 5 December 2014 / Accepted: 5 December 2014 / Published online: 21 December 2014

Abstract The genome of some vole rodents contains large blocks of heterochromatin coupled to the sex chromosomes. While the DNA content of these heterochromatic blocks has been extensively analyzed, little is known about the epigenetic modifications controlling their structure and dynamics. To better understand its organization and functions within the nucleus, we have compared the distribution pattern of several epigenetic marks in cells from two species, *Microtus agrestis* and *Microtus cabrerae*. We first could show that the heterochromatic blocks are identifiable within the nuclei due to their AT enrichment detectable by DAPI staining. By immunostaining analyses, we demonstrated that enrichment in H3K9me3 and HP1, depletion of DNA methylation as well as H4K8ac and H3K4me2, are major conserved epigenetic features of this heterochromatin in both sex chromosomes. Furthermore, we provide evidence of transcriptional activity for some repeated DNAs in cultivated cells. These transcripts are partially polyadenylated and their levels are not altered during mitotic arrest. In summary, we show here that enrichment in H3K9me3 and HP1, DNA demethylation, and transcriptional activity are major epigenetic features of sex heterochromatin in vole rodents.

Keywords Heterochromatin · Microtus · Sex chromosomes · DNA methylation · Repeat DNA transcription

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Introduction

In higher eukaryotes, the nuclear genome is compartmentalized into distinct chromatin territories to facilitate regulation of different functions such as DNA replication, transcription, or DNA repair. Two higher-order chromatin structures are typically observed, euchromatin and heterochromatin, which are defined by a complex interplay between condensation state, chromatin modifications, associated proteins, and transcriptional activity, all referred to as epigenetic marks (Richards and Elgin 2002; Francastel et al. 2000; Jost et al. 2012).

Euchromatic regions are mainly depleted of methylated DNA, enriched in specific methylated histones H3K4, H3K36, or H3K79, and present a high level of histone acetylation (Bártová et al. 2008). These marks determine mostly the less condensed and transcriptional active status of euchromatin.

Conversely, heterochromatin refers traditionally to chromosomal regions which are regularly and frequently observed to become heterochromatic (Brown 1966). Two types exist, facultative and constitutive heterochromatin. Facultative heterochromatin corresponds to developmentally silenced regions distinguished by specific enrichment in histone H3K27 trimethylated (H3K27me3), with the inactive X chromosome in mammals being one of the most prominent examples (Lyon 1961; Plath et al. 2003). In contrast to facultative heterochromatic regions, constitutive heterochromatin is equally present on both homologous chromosomes and responds in the same way in both during development, being in almost all cases C-band positive and late replicating. Constitutive heterochromatin exhibits an intricate chromatin landscape marked by enrichment in a set of histone modifications typically trimethylation of H3K9 (H3K9me3) and H4K20 (H4K20me3) and histone hypoacetylation (Kouzarides 2007). In addition, an important non-histone component is the "chromodomain-containing

heterochromatic protein" (HP1), which recognizes and binds to histone H3K9me3 (Kwon and Workman 2008). In mammals, HP1 protein presents three isoforms (HP1α, HP1β, and HP1γ) highly similar in amino acid sequence and structural organization but with particular differences in their cellular distribution (Minc et al. 1999; Li et al. 2002; Agarwal et al. 2007). The interplay between HP1 isoforms and H3K9me3 determines the establishment of a high-order structure at heterochromatin, which is further modulated by other chromatin-modifying factors, such as histone H4 acetylation and an intermediary of RNA (Cowell et al. 2002; Maison et al. 2002; Muchardt et al. 2002; Prasanth et al. 2010). This complex regulation has led some authors to hypothesize that HP1 association at heterochromatin is determined by multiple chromatin and protein interactions of which H3K9me3 is the best characterized. In fact, several studies have demonstrated that H3K9me3 is neither sufficient nor necessary for HP1 recruitment at heterochromatin (Cowell et al. 2002; Prasanth et al. 2010).

Another hallmark of constitutive heterochromatin is a high density of DNA methylation. Cytosines from CpG dinucleotides are methylated by cytosine-C5-specific DNA methyltransferases (Goll and Bestor 2005). Methylated DNA is subsequently recognized in mammals by the methyl CpG binding domain (MBD) protein family, whose founding member is "methyl CpG binding protein 2" (MeCP2) (Brero et al. 2006). These proteins bind to methylated DNA through their MBD domain and accumulate consequently at heterochromatic regions (Nan et al. 1993; Brero et al. 2005). DNA from (peri)centromeric and telomeric regions is preferentially methylated and is a crucial factor for (peri)centromere clustering during mouse cell differentiation (Barbin et al. 1994; Montpellier et al. 1994; Brero et al. 2005). DNA methylation is intermingled with the rest of epigenetic marks at the core histones and frequently overlaps with H3K9me3-enriched regions (Bártová et al. 2008).

At the DNA sequence level, constitutive heterochromatin is mostly composed of repeats. The unique conserved feature is their repeated nature as they are extremely variable in nucleotide sequence, length, and/or complexity even among related species. Satellite DNAs are the most common sequences but other types of repeats such as retrotransposons or large complex repeats are also described (Henikoff and Dalal 2005; Yan and Jiang 2007). Traditionally, these bulks of repeated sequences have been considered as junk, transcriptionally inert DNA devoid of function due to their heterochromatinized status. However, more and more evidence is accumulating in support of a functional role within different biological contexts based mainly on the existence of heterochromatin-derived transcripts. Thus, examples of transcripts derived from satellite DNAs have been reported in some species of yeasts, plants, insects, or animals, showing a high diversity in size, structure, or abundance (Grewal and Elgin, 2007; Neumann et al. 2007; Usakin et al. 2007; Pezer and Ugarkovic 2008; Bouzinba-Segard et al. 2006; Wong et al. 2007). Furthermore, those transcripts are regulated in a complex manner during different biological contexts, such as cell differentiation and proliferation, stress, cell cycle, or developmental stage (Rizzi et al. 2004; Bouzinba-Segard et al. 2006; Lu and Gilbert 2007; Shi et al. 2007; Valgardsdottir et al. 2007; Probst et al. 2010; Zhu et al. 2011; Pezer and Ugarkovic 2012). A major question emerging is if repeat-derived transcripts are central to the process of heterochromatin assembly and formation in higher eukaryotes as revealed for the yeast model *Schizosaccharomyces pombe*. Interestingly, in mouse, there is evidence that the protein Dicer, one key member of the RNAi pathway, is mandatory for repression of centromeric transcripts (Kanellopoulou et al. 2005). Alternative mechanisms still not well defined involving long noncoding RNAs (ncRNAs) are seem required for maintaining of pericentric heterochromatin (Maison et al. 2002; Wang et al. 2006).

In mammals constitutive heterochromatin is mostly arranged at (peri)centromeric regions of chromosomes. As a result, most studies on epigenetic profiling of heterochromatin have focused on the (peri)centromeric chromatin. Vole rodents (subfamily Arvicolinae) are a remarkable exception as, in addition to the normal distribution at pericentromeric regions, a bulk of heterochromatin is coupled to both sex chromosomes (reviewed in Marchal et al. 2003). This sex heterochromatin is visualized as two large blocks on the X and Y chromosomes in metaphase preparations of some species from the genus *Microtus*. Previous studies in *Microtus agrestis* showed that the degree of condensation of the heterochromatic blocks in interphase cells varies significantly during early embryogenesis and between adult cell types and cultured cells (Sieger et al. 1970; Lee and Yunis 1970, 1971; Schwarzacher 1976; Sperling et al. 1985).

Multiple analyses of the DNA content of heterochromatin in these species have revealed a diverse collection of sequences, i.e., several satellite DNA families (Modi 1992; Fernández et al. 2001; Acosta et al. 2009), interstitial telomeric sequences (ITS) (Rovatsos et al. 2011), retroelements (L1 and non-L1) (Marchal et al. 2006; Acosta et al. 2008; Neitzel et al. 1998, 2002), large complex repeats (Marchal et al. 2004a; Kalscheuer et al. 1996; Modi et al. 2003), and pseudogenes (Marchal et al. 2008). The repeat content is particularly complex and variable for the sex heterochromatic blocks, which have rapidly and independently evolved in each species (Marchal et al. 2004b; Sitnikova et al. 2007; Acosta et al. 2011). Despite of extensive DNA screening, little is known about the epigenetic modifications controlling the structure and dynamics of these atypical pieces of heterochromatin. To better understand their function and organization within the nucleus, we have analyzed the pattern of several histone modifications, HP1 distribution, and DNA methylation in cells from two different vole species,

Microtus cabreare and *M. agrestis*. Furthermore, we provide evidence for the existence of transcripts derived from some DNA repeats heavily amplified at the sex heterochromatic blocks. Our study reveals that H3K9me3 and HP1beta deposition, DNA demethylation and transcriptional capacity are major epigenetic determinants of this type of heterochromatin.

Material and methods

Cell culture and transfection

Permanent fibroblast cell lines from male individuals of *M. agrestis* and *Microtus cabrerae* species were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum and 1 % of antibiotics (penicilin+streptomycin) in 5 % CO_2 atmosphere at 37 °C. Cells used in immunofluorescence were plated and grown on glass coverslips (15-mm diameter). Transfection of cells was carried out by the PEI method as described in Casas-Delucchi et al. (2012). Expression vectors employed in transfections were YFP-tagged fusion of MBD domain from MeCP2 protein cloned into pEGFP-C1 vector (Brero et al. 2005) and Cherry-tagged fusion of HP1β cloned into pEGFP-C1 vector.

FISH

Mitotic preparations from *M. agrestis* and *M. cabrerae* cell cultures were obtained following standard protocols previously described (Marchal et al. 2004b). For hybridization in *M. agrestis*, we employed as probe the plasmid pMAHae2-2 (Neitzel et al. 1998), which contains a repeat fragment of 2.9 Kb from the heterochromatic blocks of *M. agrestis* cloned into pT7T3 vector. Labelling was performed with biotin-16-dUTP (Roche) by random primers method. In *M. cabrerae* preparations, we performed chromosome painting using as probes the heterochromatic blocks from the X and Y chromosomes obtained by microdissection. In particular, we microdissected the whole Y chromosome, which is nearly complete heterochromatic, and the large arm of the X chromosome, totally heterochromatic, which were further amplified and labeled by DOP-PCR method as described in Marchal et al. (2004b). Images were captured using an Axiovert 200 microscope with a ×63/1.4 Plan-Apochromatic oil objective (Zeiss).

Immunofluorescence

Cells were grown on glass coverslips, fixed in 4 % paraformaldehyde for 10 min at RT and permeabilized for 20 min at RT in 0.5 % Triton X-100/PBS. Immunofluorescence staining was performed in 4 % BSA/PBS for 1 h at RT (primary antibodies) and for 45 min at RT (secondary antibodies). The following primary antibodies were used: rabbit anti-H3K9me3 1:200 (Upstate), rabbit anti-H4K8ac 1:200 (Upstate), and rabbit anti-H3K4me2 1:800 (Biomol). Secondary antibody was anti-rabbit-alexa488 1:200 (Invitrogen). For immunodetection of DNA methylation, we employed an anti-5-methylcytidine antibody (Eurogentec) according to the protocol described in Rens et al. (2010). Briefly, metaphase preparations were denaturated using 70 % Formamide at 65 °C during 1 min, incubated with primary and secondary antibodies diluted at appropriated conditions in 0.01 % of TKCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA pH 8.0, 0.1 mM Triton X-100), and further fixed in 4 % paraformaldehyde during 15 min. In all our immunostainings, DNA was visualized with DAPI 1 μg/ml (Sigma-Aldrich) before final montage with Vectashield antifade (Vector laboratories). Images were captured with an Axiovert 200 microscope with a 63/1.4 Plan-Apochromatic oil objective (Zeiss). Grayscale images were pseudocolored and merged using ImageJ.

RNA isolation, RT-PCR, and qRT-PCR

For transcriptional analyses, we employed both tissue and cell culture samples from *M. agrestis*. Tissues analyzed were spleen, liver, ovary, testis, brain, and kidney of one male and one female individual of *M. agrestis*. Cell culture samples were processed into different pools that differ in the amount of mitotic cells. For that, one cell culture was enriched in mitosis by colcemid incubation during 2 h and subsequent mitotic shake-off while the rest of cell cultures were not treated and included a low rate of mitotic cells. A small cell fraction from each culture was used for determining the mitotic index by microscopy analyses on conventional cytogenetic preparations, which was 29 % for the mitotic-arrested sample and 2.2–4 % for the non-arrested ones (n=1000). RNA was purified using "RNeasy Mini" kit (Qiagen). To eliminate traces of coextracted DNA, purified RNA was always treated during 2 h with up to 0.5 units of DNase I (RQ1 Rnase-Free Dnase, PROMEGA) per microgram of RNA prior to further reverse transcription.

RNA was transcribed into cDNA using "Transcriptor First Strand cDNA Synthesis Kit" (Roche). cDNA synthesis conditions were 10 min at 25 °C, 60 min at 55 °C, and 5 min at 85 °C. The primers employed were either random or oligo-dT primers provided by the kit. In addition, we also used in the cell culture analyses the primers Hae2-for (AGCTGAATAA CTAAGAAAGAAT) and Hae2-rev (TCACCTATCCTGTA TCTATCTC), specifically complementary to either forward or reverse strand of pMAHae2 repeated DNA. For better comparison of transcript levels in cell culture analyses, equal number of cells (10^6) and equal amount of purified RNA were always employed in the reactions. Additionally, to investigate and compare the nature of the transcripts, each RNA sample obtained from the described cell pools was further divided into

four aliquots that were reverse transcribed in parallel using the four mentioned primers. Reactions in which reverse transcription enzyme was omitted (RT-) as well as reactions with no added template were performed as controls for DNA contamination and cross-contamination between samples. cDNA samples were further amplified by PCR (RT-PCR) using the following primer pairs: 11/3-for (ACTTTGGGACACAGAA CTCAT) and 11/3-rev (TTACAACGTGTGTCAAACTTTA), specific for retroelement pMA11/3 (Neitzel et al. 2002); L1-for (ACCTAAGAAACAAGCAGGTGT) and L1-rev (GGCATAGATATTCAGGATTGAG), specific for an L1 fragment of *Microtus* genome (Marchal et al. 2006); Hae2-for (AGCTGAATAACTAAGAAAGAAT) and Hae2-rev (TCACCTATCCTGTATCTATCTC), specific for pMAHae2 repeated DNA of *M. agrestis* (Neitzel et al. 1998); GAPDH-for (TGCACCACCAACTGCTTAGC) and GAPDH rev (GGCATGGACTGTGGTCATGAG), specific for GAPDH gene; and Bact-for (CCAACCGTGAAAAGATGACC) and Bact-rev (TCGGTCAGGATCTTCATGAG), specific for beta-actin gene. Primers were designed using OLIGO 4.1 software. Amplifications were carried out for 35 cycles under the following conditions: 5 min at 94 °C, 30 s at 55 °C, and 10 s at 72 °C, with a final extension of 5 min at 72 °C.

For quantitative real-time PCR (qRT-PCR) cDNAs, samples were amplified using "SsoFast Evagreen Supermix" kit (BIO-RAD) and the primers above described. All reactions were performed on "CFX Real-Time PCR system" (BIO-RAD) in three replicates in a final volume of 10 μl, under following reaction conditions: 95 °C 1 min, 95 °C 10 s, 55 °C 10 s, and 60 °C 10 s, for 40 cycles. Non-template and RT- controls were included. The specificity of the amplifications was confirmed by analyses of melting curves and, when required, by conventional agarose electrophoresis. The data were analyzed using the comparative Ct method (ΔΔCt). cDNA from cell culture samples without enrichment in mitotic cells served as reference for comparison and B-actin gene was used as endogenous control for normalization. We never observed any significant variation in the expression of this housekeeping gene in our analyses.

Results

Identification by DAPI staining of heterochromatic blocks during interphase

To determine if the two bright foci observed in interphase nucleus of *M. agrestis* and *M. cabrerae* cells stained with DAPI correspond to the sex heterochromatin, we performed FISH analyses using specific probes for the heterochromatic blocks of each species. Thus, in *M. agrestis*, we employed the repeated DNA pMAHae2, which is exclusively distributed along the heterochromatic blocks of both sex chromosomes (Neitzel et al. 1998). When used on interphase cells, this probe always colocalized with both regions strongly stained by DAPI, confirming our previous assumption (Fig. 1a). For *M. cabrerae*, we used painting probes specific for the heterochromatic blocks of the X and Y chromosomes as the DNA composition of the sex heterochromatin is different within

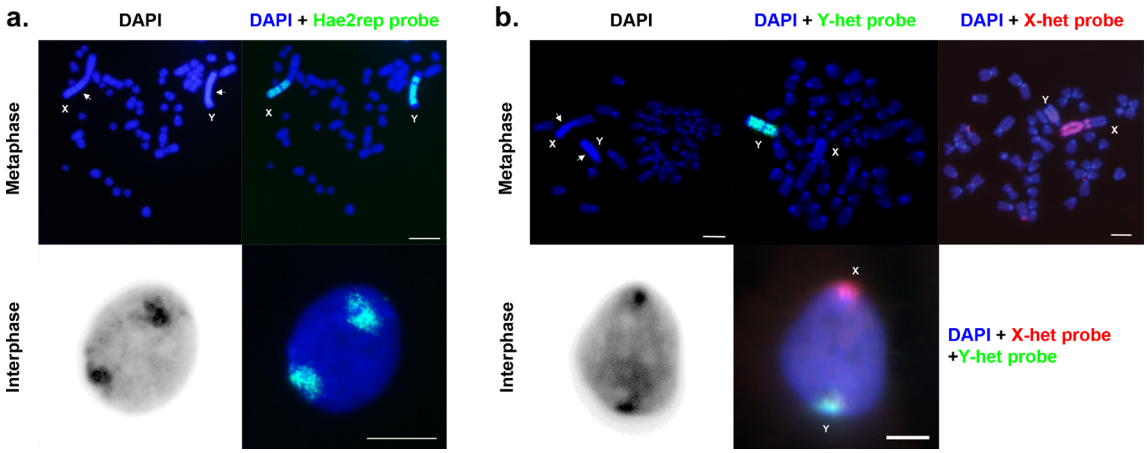

Fig. 1 Distribution of heterochromatic blocks of sex chromosomes in *Microtus* cells. **a** Metaphase chromosomes (*top*) and interphase nuclei (*bottom*) from *M. agrestis* were analyzed by FISH with a probe obtained from the repeated sequence pMAHae2. Images shown correspond to metaphase DAPI staining (*top left*) and merge with the probe signal (*top right*). *Arrows* point to the heterochromatic blocks coupled to the X and Y chromosomes. DAPI image of interphase nuclei is shown in *grey scale* and inverted to facilitate visualization of the heterochromatic blocks (*bottom left*), both positive hybridized with the probe (*lower right*). **b** Metaphase chromosomes (*upper*) and interphase nuclei (*lower*) from *M. cabrerae* were analyzed by FISH with painting probes from the heterochromatic blocks of the Y and X chromosomes. Images shown correspond to metaphase DAPI staining (*top left*), and merge with probes from the heterochromatic blocks of the Y (*top center*) and the X chromosome (*top right*), respectively. *Arrows* point to the heterochromatic blocks coupled to the X and Y chromosomes. DAPI image of interphase nuclei is shown in *grey scale* and inverted to facilitate visualization of the heterochromatic blocks (*lower left*), which were simultaneously hybridized with both probes (*bottom right*). *Scale bar*= 5 μm

chromosomes (Marchal et al. 2004b) (see "Material and methods"). The specificity of our painting probes was first confirmed by FISH on metaphase chromosomes and when hybridized simultaneously in interphase cells each of the Y- and X-heterochromatic probes painted specifically one of the two bright foci observed by DAPI staining (Fig. 1b). On the basis of these observations, we conclude that the two bright regions observed by DAPI in interphase cells of *M. cabrerae* and *M. agrestis* correspond, as expected, to the heterochromatic blocks of the sex chromosomes. Hence, hereafter, we made use of the DAPI staining intensity to identify the heterochromatic blocks of the sex chromosomes.

Distribution of chromatin marks in heterochromatic blocks during interphase

Next, we analyzed by immunofluorescence the overall distribution of several histone modifications in cells from both species. This analysis allowed us to determine the pattern of enrichment/depletion for each specific epigenetic modification at the heterochromatic blocks. The modifications investigated included H4K8-acetylation (H4K8ac) and H3K4-dimethylated (H3K4me2), both typical marks of transcriptionally active regions and, therefore, frequently enriched at euchromatin. In *M. agrestis*, both marks were excluded from the DAPI-stained regions (Fig. 2), which corresponds to the heterochromatic blocks of both sex chromosomes as demonstrated above. The rest of the nucleus exhibited signal of variable intensity for both histone marks. Central regions depleted of H4K8ac signal correspond to nucleoli, as identified by phase contrast microscopy (not shown). Identical results were found when we performed the equivalent analyses on *M. cabrerae* cells (Fig. 2). Our results demonstrate the depletion of these two typically euchromatic epigenetic modifications from the heterochromatic blocks of the sex chromosomes of *Microtus*.

We further investigated the distribution pattern of two epigenetic marks typical for heterochromatin, histone H3K9me3 and HP1-beta accumulation. The distribution of both epigenetic marks was determined simultaneously by immunostaining with an antibody against H3K9me3 in *Microtus* cells expressing mCherry-tagged HP1-beta (Fig. 3), as our previous attempts to localize HP1-beta by immunostaining using different commercial antibodies were unsuccessful. In *M. agrestis* cells, we observed a strong signal for both marks overlapping on the heterochromatic blocks visualized by DAPI. Similar results were obtained in *M. cabrerae* cells as two intense signals for each mark, H3K9me3 and HP1-beta, colocalized with the DAPI-positive regions (Fig. 3). From these analyses, we conclude that the heterochromatic blocks of both species are enriched for two of the most prominent chromatin modifications typical for constitutive heterochromatin, H3K9me3 and HP1-beta.

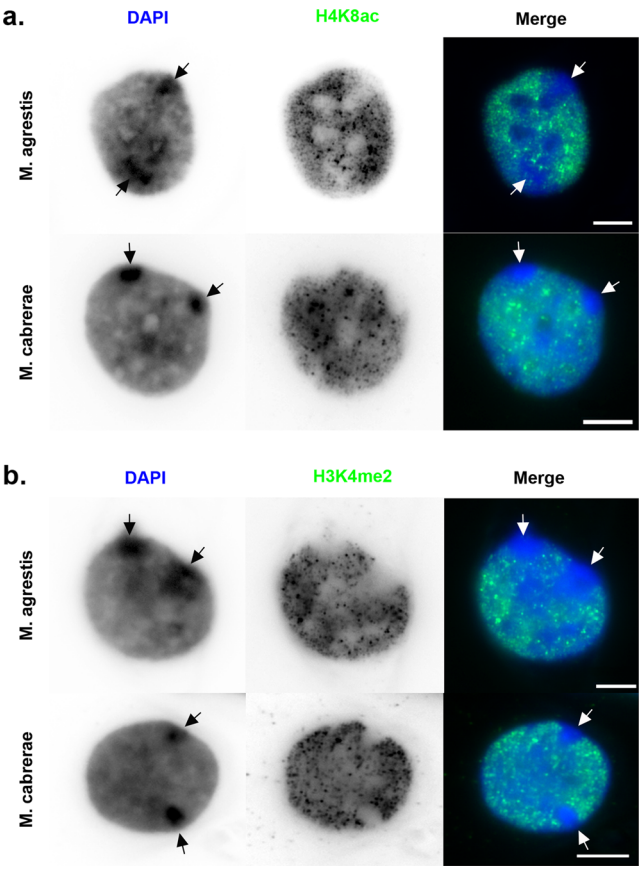

Fig. 2 Distribution of euchromatin marks in *Microtus* cells. Cells from *M. agrestis* (*top*) and *M. cabrerae* (*bottom*) were analyzed by immunostaining against H4K8ac (**a**) and H3K4me2 (**b**). DAPI (*left*), protein (*center*), and merge (*right*) signals are shown. The heterochromatic blocks from the sex chromosomes are visualized as two DAPI intense signals in each cell, signalized by *arrows*. *Scale bar*=5 μm

Heterochromatic blocks are depleted of DNA methylation

The long-range distribution of DNA methylation in *Microtus* was analyzed by immunostaining on metaphase chromosomes using an antibody against 5-methylcytosine (5mC) (Fig. 4a). In both species, we obtained similar results, with the heterochromatic blocks of both X and Y chromosome strongly depleted of signal for the 5mC antibody in relation to the adjacent euchromatic regions from the same chromosome and to the rest of chromosomes. Centromeric regions, which also present a more intense DAPI staining, showed an equally weak signal compared with the adjacent euchromatic regions. Thus, these results suggest that heterochromatin from both sex blocks and centromeric regions are hypomethylated in cells from these *Microtus* species.

To confirm and extend these results, we analyzed some of the proteins involved in recognition of methylated DNA. Methyl CpG binding protein 2 (MeCP2) binds DNA and has a preference for methylated sequences, consequently accumulating in cells at heterochromatin (Jost et al. 2011). Here, we

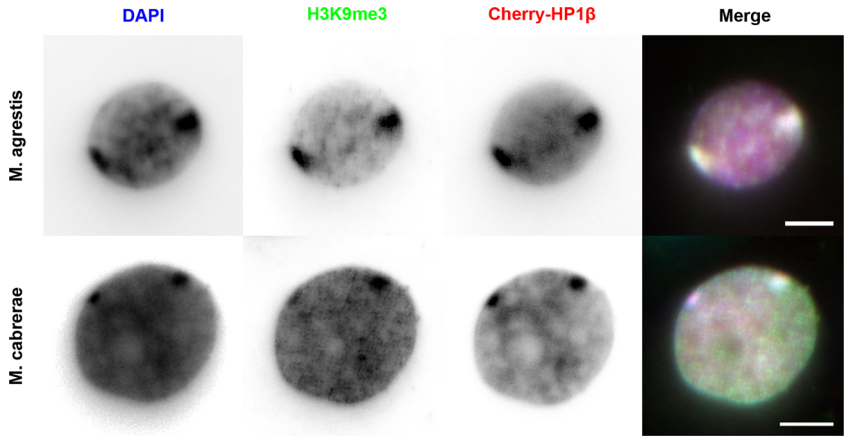

Fig. 3 Distribution of marks from constitutive heterochromatin in *Microtus* cells. Cells from *M. agrestis* (*top*) and *M. cabrerae* (*bottom*) expressing the fusion protein mCherry-HP1β were analyzed by immunostaining against H3K9me3. DAPI (*far left*), H3K9me3 (*mid-left*), mCherry-HP1β (*mid-right*) and merge (*far right*) signals are shown. *Scale bar*=5 μm

transfected cells with an expression construct coding for YFP-tagged MeCP2 protein (Brero et al. 2005) to detect 5mC in interphase cells. As a control for our experiment we also transfected mouse myoblasts, where we observed the expected colocalization of the exogenously expressed MeCP2 protein with heterochromatic chromocenters, visualized as DAPI intense foci in interphase cells (data not shown). Contrary, in cells from both *Microtus* species, the signal of MeCP2 was distributed over the whole nuclei but largely excluded from the heterochromatic blocks (Fig. 4b). Taken together, our results demonstrate that DNA methylation is not an epigenetic mark characteristic of neither the heterochromatic blocks nor the (peri)centromeric regions of *Microtus* species.

Heterochromatin transcription

Previous studies have reported the occurrence of transcriptional activity for the heterochromatic blocks of *M. agrestis* in cultured cells (Sperling et al. 1987, Neitzel et al. 2002). Here, we have characterized the transcriptional profile of several repeat sequences from this species both in tissues and cell cultures using RT-PCR methods. First, we performed RT-PCR using primers specific for pMA11/3, LINE-1 and pMAHae2 repeats on cDNA samples obtained from different tissues of male and female adult individuals (liver, brain, spleen, kidney, testis, and ovary) (Fig. 5a). When using primers specific for pMA11/3 sequence, a retroelement interspersed in the genome but locally enriched in the heterochromatic blocks (Neitzel et al. 2002), we obtained a band of the expected size in all male and female samples. The intensity of such band was variable within the analyzed samples. We also analyzed a LINE-1 fragment heavily amplified on the heterochromatic blocks (Acosta et al. 2008), and we observed RT-PCR amplification of the expected size in all samples. In this case, the fragment was more equally amplified among the samples compared to the previous sequence. For pMAHae2 sequence, a complex repeated DNA exclusively amplified in the heterochromatic blocks (Kalscheuer et al. 1996), we observed amplified fragments of the expected size and variable intensity in all male samples while in female samples, only brain, spleen, and kidney produced the expected band with variable intensities. The lack of transcription in some female tissues could be related to the extremely low rate of transcription noticed for this repeated sequence (see below), possibly under the

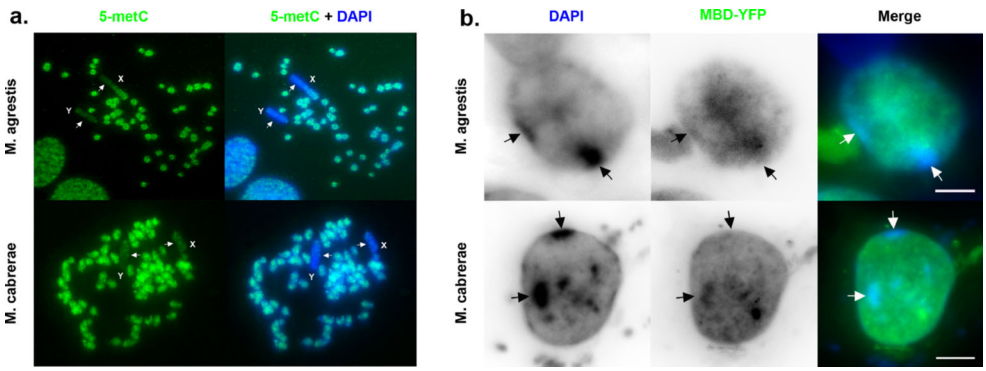

Fig. 4 DNA methylation pattern in *Microtus* cells. **a** Mitotic preparations from *M. cabrerae* and *M. agrestis* were analyzed by immunostaining against the modified nucleotide 5-methylcytidine (5mC, green). DNA was stained with DAPI (*blue*). In both cases, heterochromatic blocks from the large X and Y chromosomes (pointed by *arrows*) are depleted of 5mC signal. **b** Cells from *M. cabrerae* and *M. agrestis* expressing YFP-tagged MBD domain of MeCP2 protein. DAPI (*left*), MeCP2-YFP (*center*), and merge (*right*) signals are shown. Notice that the heterochromatic blocks, visualized in the nuclei as DAPI intense signal, are depleted of signal. *Scale bar*=5 μm

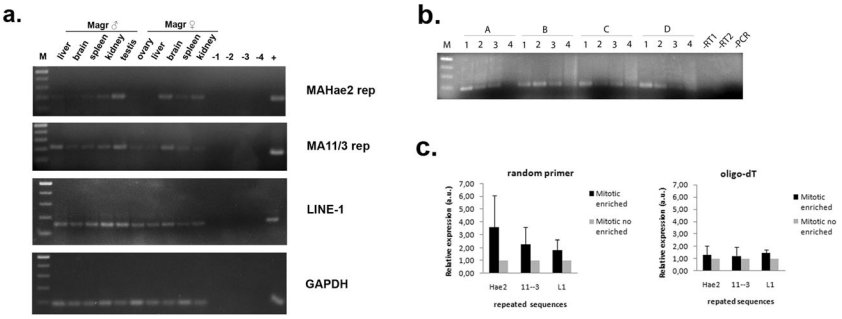

Fig. 5 Transcriptional analyses of repeat DNAs in *M. agrestis* cells. **a** RT-PCR using RNA samples obtained from the indicated tissues of male and female adult individuals. The sequences analyzed were as follows: MAHae2rep, a complex repeat sequence exclusively located at the heterochromatic blocks; MA11/3 rep, an active retroelement widely distributed in the genome and heavily amplified on the heterochromatic blocks; LINE-1, a fragment of a long interspersed nuclear element; and GAPDH, an endogenous gene used as reference for the cDNA synthesis. The primers used in each amplification produced a specific band of the expected size. *M* molecular marker (the observed bands range from 100 to 500 bp). *−1*, control without RNA. *−2*, control without RT enzyme (testis sample). *−3*, control without RT enzyme (ovary sample). *−4*, PCR negative control. *plus sign*, PCR-positive control. **b** RT-PCR with primers specific for pMAHae2 repeat same as in (**a**) using RNA samples obtained from four independent cell cultures of *M. agrestis* (*A–D*). Samples *A*, *B*, and *D* were prepared from conventional cell cultures with a low rate of mitotic cells (mitotic index estimated were 3.8, 2.2, and 4 %, respectively). Sample C was enriched in mitotic cells (mitotic index 29 %) by colcemid incubation during 2 h and subsequent mitotic shake-off. RNA samples were reverse transcribed using four different primers: random primers (*1*), oligo-dT (*2*), and the specific primers Hae2-for (*3*) and Hae2-rev (*4*), complementary to either forward or reverse strand of pMAHae2 repeated DNA. *–RT1*, control without RNA. *–RT2*, control without RT enzyme (sample *A*). *–PCR*, PCR negative control. **c** Relative comparison of repeated transcript levels in cell culture samples enriched in mitotic cells (prepared as described in *B*, mitotic fraction 29 %) and in proliferating cells with lower rate of mitosis (mitotic fraction 3.9 %). The repeated DNAs analyzed are the same as in *A*. Transcript levels were relatively compared by qRT-PCR using B-actin as endogenous control and mitotic non-enriched data as reference value. The data represent the average and SD from two independent experiments. The analyses were done independently for cDNAs synthesized with either random or oligo-dT primers

threshold for RT-PCR detection in some cases. Finally, we did not observe amplification on these samples in RT-PCRs with primers specific for McaY851 sequence (data not shown), a repeated DNA specific for the Y heterochromatin of vole species but scarcely present in *M. agrestis* genome (Marchal et al. 2004a). All cDNA samples produced the expected amplification for the loading control GAPDH gene. Importantly, the lack of a band in the negative controls ensured that the amplification result from reverse transcribed RNA and not from residual genomic DNA. Taken together, these results indicate that the existence of transcripts from different types of repeated sequences is a rather common feature in adult tissues from *M. agrestis*.

We next found by RT-PCR the occurrence of transcription for the repeated sequences described above in cultured fibroblasts cells from this species (data not shown; the apparent conflict between our results about pMAHae2 transcription and those of Kalscheuer et al. 1996 is discussed later). To get more insights into the transcriptional activity of the heterochromatic blocks, we analyzed the nature of the transcripts produced from pMAHae2 sequences and whether their levels are altered during mitotic arrest. Using the same primers for pMAHae2 sequence as in Fig. 5a, we performed RT-PCR analyses on RNA samples obtained from cell culture pools differing in the amount of mitotic cells (Fig. 5b, samples A–D, see "Material and methods"). Our analyses revealed first amplification of the expected size in all RNA samples reverse transcribed with either random or oligo-dT primers. When using specific primers, we observed amplification in all but one cDNA samples obtained with primer Hae2-for, but no clear band was detected in all cDNA samples synthesized with primer Hae2-rev. In all cases, we observed the expected amplification for the loading control gene GAPDH (data not shown). The same results were obtained for the different cell culture pools analyzed (samples A–D). These results suggest that transcripts from pMAHae2 repeats are partially polyadenylated and preferentially produced from one DNA strand in both mitotically arrested and non-arrested cells of this species.

Finally, we analyzed if the transcription levels of pMAHae2, pMA11/3, and LINE-1 sequences are altered during mitotic arrest, since cell proliferation seems to be an important factor regulating the transcription of heterochromatic sequences in some species (Lu and Gilbert 2008). We performed a relative comparison by qRT-PCR method using the cellular pools described above, which were differentially enriched in mitotic cells by colcemid arrest. We compared the overall transcription of pMAHae2 repeat, pMA11/3 retroelement, and LINE-1 sequences in cDNA samples prepared with either random primers or oligo-dT. Our results showed no statistically significant differences in the transcript levels of any of the repeat sequences analyzed (Fig. 5c). Moreover, in all the samples analyzed, the transcript levels were of extremely low abundance for pMAHae2, being ≈500–1000-fold less abundant than B-actin transcripts, while pMA11/3 and LINE-1 transcripts showed a reduction of ≈100-fold and 30-fold compared with B-actin levels,

respectively. Taking into account the extremely large differences in copy number between the repeated DNAs and B-actin, our results suggest that the transcript levels of the repeated sequences analyzed here are strongly reduced. Furthermore, the repeat transcript levels are not altered during mitotic arrest in *M. agrestis* cells.

Discussion

The genome of some vole rodents is characterized by an enrichment of heterochromatin arranged mostly into two large blocks coupled to the X and Y chromosomes. In this work, we have performed a comparative investigation of the most prominent epigenetic modifications associated with heterochromatin in general, such as histone hypoacetylation and methylation, HP1 association, DNA methylation, and the occurrence of repeat-derived RNAs.

Our results clearly demonstrate that heterochromatic blocks from the X and Y chromosomes of *M. agrestis* and *M. cabrerae* are both enriched in H3K9me3 and HP1-beta and depleted of H4K8 acetylation and H3K4me2. This corresponds to the typical pattern for a (constitutive) heterochromatic domain in higher eukaryotes. Our results confirm and further extend previous findings of Shevchenko et al. (2009), which analyzed female cells from another *Microtus* species, *Microtus rossiameridionalis*, and demonstrated that the heterochromatic block of the X chromosome is enriched in both H3K9me3 and HP1-beta and depleted of H3K4me2. In combination with that study, our results in *M. cabrerae* and *M. agrestis* demonstrate that those chromatin modifications are highly conserved features of the heterochromatic blocks of these rodent species. Furthermore, our characterization of the distribution pattern of these epigenetic marks during interphase complements that previous study, which was focused on metaphase. That suggests that in *Microtus* species, these chromatin modifications are stable throughout the cell cycle, although temporal changes during particular cell cycle stages cannot be excluded. The dynamics of epigenetic modifications during the cell cycle are variable. For instance, HP1-beta deposition in mouse and human heterochromatin varies in a cell-cycle-dependent manner, with most of it being redistributed from chromatin to the cytoplasm during metaphase (Minc et al. 1999; Serrano et al. 2009). However, H3K9me3 remains associated with constitutive heterochromatin during S-phase and also in mitosis in mouse cells (Cowell et al. 2002).

Several studies have pointed to the importance for HP1 binding to trimethylated histone H3K9 in the nucleosomes. HP1 interacts with the histone methyl-transferase Suv(3)9h1, which is responsible for methylation at H3K9 creating thus a binding site for HP1 that serves as an assembly platform for other chromatin-modifying factors involved in stabilization and spreading of heterchromatin, i.e., histone deacetylases (Aagaard et al. 1999; Rea et al. 2000; Grewal and Jia 2007). Our observations allow speculation about the possibility that the interplay between H3K9me3 and HP1 deposition might be similarly essential for the structural conformation of the heterochromatic blocks in *Microtus* cells.

Here, we present data indicative that DNA hypermethylation is not an epigenetic mark defining the heterochromatic blocks in proliferating cells as (i) a very weak signal was detected along these regions when performing immunostaining with an antibody against 5mC on metaphase chromosomes and (ii) MeCP2, one of the key proteins involved in recognition of methylated DNA, is not enriched at heterochromatic blocks. Our results confirm also previous findings based on restriction analyses using methylation-sensitive/insensitive enzymes on *M. agrestis*-cultured cells, which showed hypomethylation of one of the major sequences composing the heterochromatic blocks, pMAHae2 repeat (Kalscheuer et al. 1996). In general, repeat sequences from constitutive heterochromatin are AT rich and seem to be subjected to hypermethylation, easily detectable by using 5mC-specific antibodies (Barbin et al. 1994; Montpellier et al. 1994; Miniou et al. 1994). It is therefore generally accepted that DNA hypermethylation correlates with heterochromatin, being a crucial factor for its structural conformation (Lehnertz et al. 2003; Brero et al. 2005). However, our present study revealed that heterochromatic blocks from *Microtus* rodents are enriched in several epigenetic marks typical of heterochromatin (H3K9me3, HP1) but hypomethylated and depleted of proteins binding to 5mC. Interestingly, in mouse cells defective for the Capthesin L gene, heterochromatin displays reduced levels of H3K9me3 and HP1 marks but is normally condensed and presents a hypermethylated DNA pattern (Bulynko et al. 2006). These authors propose that methylation of both histones and DNA could be functionally uncoupled and operate on independent pathways in heterochromatin formation. To gain insights into this hypothesis, it will be of interest to further investigate the exact role of DNA methylation on heterochromatin conformation in *Microtus* species.

In this work, we proved the existence of transcripts derived from some repeat sequences of *M. agrestis* in both tissues and cell culture. Furthermore, our analyses using proliferating cultivated cells showed no apparent difference for the transcription rate of these sequences after mitotic arrest by colcemid incubation. The present results extend previous studies noticing the transcriptional activity for the heterochromatic blocks of this species. First evidences came from analyses in cultivated fibroblasts which showed a DNase I sensitivity for the heterochromatic blocks similar to the euchromatin as well as positive labelling after in situ hybridization with total cellular RNA (Sperling et al. 1985, 1987). A further study indicated, in accordance to our results, the existence of

transcripts derived from the sequence pMA11/3, a retroelement located mostly but not exclusively in the heterochromatic blocks of *M. agrestis* (Neitzel et al. 2002). However, another previous work did not detect transcription from the element pMAHae2, a large complex repeat, which is heavily amplified in both heterochromatic blocks (Kalscheuer et al. 1996). The apparent discrepancies between our data about pMAHae2 transcription and the previous one of Kalscheuer et al. (1996) are likely explained by the used methods. The previous studies based transcript identification on hybridization techniques as Northern blot or in situ hybridization, whereas in our study we have applied RT-PCR, a more sensitive method. Accordingly, our QRT-PCR data indicate that transcript levels of pMAHae2 are extremely low, emphasizing why transcription is only detectable by RT-PCR methods. A similar scenario has been documented in human cells, where initial Northern studies revealed the existence of Sat3 RNAs only after thermal stress, whereas by RT-PCR was further detected a basal expression of Sat 3 repeats even in unstressed cells (Valgardsdottir et al. 2007). Furthermore, since previous analyses suggested that the heterogeneous size (1–5 Kb) of pMA11/3 transcripts is consequence of read-through mechanisms (Neitzel et al. 2002; Sperling et al. 2004), we could not discard that the detected transcription of pMAHae2 sequences is caused by a similar process during RNA formation from flanking pMA11/3 elements interspersed in the heterochromatic blocks.

The existence of heterochromatic-derived transcripts has been a major question due to the challenge of their detection; it is only after the introduction of RT-PCR methods that they have been identified in a wide group of species and cell lines (Lu and Gilbert 2008). Low expression of repeated DNAs seems to be a common phenomenon documented in many organisms including vertebrates, invertebrates, and plants (reviewed in Pezer and Ugarkovic 2012). It remains an open question whether in vertebrates, a basal transcription rate is merely a cellular byproduct or an indispensable event for maintenance of heterochromatin state, as is demonstrated in yeast (reviewed in Grewal and Elgin, 2007), Interestingly, an unidentified RNA component seems to be required for maintaining of pericentric heterochromatin in mammals (Maison et al. 2002; Wang et al. 2006).

Finally, it did not escape our attention that the particular epigenetic landscape of the heterochromatic blocks of *Microtus*, that is, H3K9me3 and HP1beta deposition, DNA demethylation, and transcriptional capacity, is another feature in support of the "distinction" of this type of heterochromatin from centromeric DNA satellite-based heterochromatin. This idea was originally proposed by Neitzel et al. (1998), who defined the sex heterochromatin of these rodent species as a mammalian counterpart of the beta-heterochromatin of Drosophila based on their similarities in sequence organization and transcriptional activity. To determine if this sex heterochromatin also presents any phyisiological or functional role different from centromeric heterochromatin will be an interesting task to address in future studies.

Acknowledgments This work was supported by Ministerio de Ciencia y Tecnología of Spain (grant CGL2009-07754, cofunded by European Regional Development Fund), by Junta de Andalucía (Funding program "Ayudas a grupos de investigación," reference BIO 220) and by the Deustche Forschungsgemeinschaft grants Ca 198/7-2 and Ca 198/9-1. The authors express their gratitude to K. Sperling and H. Neitzel for providing the cell line of *M. agrestis* and to Junta de Castilla y Leon for capture permits of *M. agrestis*. We thank Anne Lehmkuhl for excellent help with cell culture. Technical and human support provided by CICT of Universidad de Jaén (UJA, MINECO, Junta de Andalucía, FEDER) is gratefully acknowledged.

Conflict of interest The authors declare no conflict of interests.

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